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Title: Breeding and Genetics of Lilacs and Hardy Hibiscus

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Lilacs are a group of ornamental trees and shrubs in the Oleaceae family consisting of 22 to 30 species. There are six series within genus *Syringa*: *Pubescentes*, *Villosae*, *Ligustrae*, *Ligustrina*, *Pinnatifoliae*, and *Syringa*. Fertility and cross-compatibility among cultivars, species, and series have yet to be formally investigated. Over three years, a cross-compatibility study was performed using elite cultivars and species of shrub-form lilacs in series *Syringa*, *Pubescentes*, and *Villosae*. We report the success of each of these combinations and the fertility estimates of viable crosses. This study is a comprehensive investigation of lilac hybridization, and the knowledge gained on cross-compatibility will aid future efforts in lilac cultivar development.

Genome size variation can be used to investigate biodiversity, genome evolution, and taxonomic relationships among related taxa. In addition, plant breeders use genome size variation to identify parents useful for breeding sterile or improved ornamentals. Reports conflict on genome evolution, base chromosome number, and polyploidy in lilac. Flow cytometry was used to estimate holoploid (2C) genome sizes in series, species, cultivars, and seedlings from parents with three ploidy combinations: 2*x* x 2*x*, 2*x* x 3*x*, and 3*x* x 2*x*. Monoploid (1C*x*) genome sizes were calculated by dividing 2C genome size by ploidy, which was confirmed in a subset of taxa using root tip microscopy. Pollen diameter was measured to

investigate the frequency of unreduced gametes in diploids and triploids. Interploid crosses between 'Blue Skies' (2*x*) and 'President Grévy' (3*x*) produced an aneuploid population with variable 2C genome sizes. One viable seedling was recovered from a cross between 'President Grévy' (3*x*) and 'Sensation' (2*x*). This near pentaploid (5*x*) seedling had a larger 2C genome size than either parent, and the largest 2C genome size currently reported in lilac. Pollen diameter measurements revealed that 'Sensation' produced 8.5% unreduced pollen. Increased ploidy may provide a mechanism for recovering seedlings from incompatible taxa in lilac breeding.

Common lilac, *Syringa vulgaris*, is an important flowering shrub that accounts for a large share of spring sales in the U.S. nursery industry. However, little research has focused on shortening generation time for lilac breeders. In a previous cross-compatibility study, observations revealed that first-year hybrid seedlings undergo a quiescent phase of growth, producing few leaves but an extensive root system. This study investigated the effects of six germination and post-germination treatments of green seed and dry, dehisced seed on seed germination and subsequent growth in lilacs. Green seed extracted 20 weeks after pollination had the highest germination rate and an increase in vegetative growth compared to controls. Our results indicate that green seed sowing may provide a new tool for shortening juvenility and reduced breeding time in common lilac.

Remontancy (reblooming) and disease resistance are two important traits in the dwarf lilacs (*Syringa pubescens*). Marker-assisted selection could prove useful at producing more disease-resistant, floriforous lilacs for future breeders. To aid future efforts at at marker discovery, genotyping-by-sequencing was applied to a bi-parental mapping population from *S. meyeri* 'Palibin' x *S. pubescens* Bloomerang® which varies for remontancy and resistance to bacterial blight. SNP-based genetic linkage maps were created for each parent, and maps will continue to be improved with further sequence data. Future efforts to phenotype the mapping population will be combined with these findings for marker-trait association.

Althea (*Hibiscus syriacus*) is an ornamental shrub prized for its winter hardiness and large colorful summer flowers. Althea are primarily tetraploids (2*n* = 4*x* = 80) with higher level polyploids reported from experiments with spindle-fiber

inhibitors. Previous studies report anatomical variation among althea polyploids, including changes in stomata size. The purpose of this study was four-fold. The first was to identify genome size and ploidy variation in althea cultivars via flow cytometry and root tip chromosome counts. The second was to create a ploidy series consisting of 4*x*, 5*x*, 6*x*, and 8*x* cytotypes using a combination of interploid hybridization and autopolyploid induction via colchicine and oryzalin. The third was to investigate the ploidy series for variation in stomatal guard cell length, stomatal density, and copy number of fluorescent rDNA signals. The fourth was to investigate segregation patterns in rDNA signals in a subset of pentaploid seedlings. Results of this study revealed ploidy differences among available cultivars. Polyploid induction and interploid hybridation were successful for producing a ploidy series that varied in stomata size, stomata density, and number of 5S and 45S rDNA signals. The rDNA loci confirmed ploidy levels in each cytotype of our ploidy series, and random segregation of rDNA loci provides evidence of random chromosome segregation in interploid hybrids of althea.

Despite its attractive, ornamental flowers, althea produces capsules with numerous, fertile seeds that germinate and cause a nuisance in production and the home landscape. Breeding for sterile forms of althea has long been a goal for *Hibiscus* breeders, yet many popular "sterile" cultivars have been reported as weedy. The purpose of this study was to evaluate female and male fertility for tetraploid and hexaploid cultivars, and to evaluate the female fertility of pentaploid seedlings resulting from 4*x* x 6*x* and 6*x* x 4*x* crosses. Self- and cross-incompatibilities were discovered, as was variation in seeds per capsule and seeds per pollination. In addition, significant differences were found among flower forms (single, semi-double, and double) for fertility estimates. Double-flowered forms had reduced female fertility, which may indicate that breeding for increased petaloid stamen may result in a reduction in female fertility. Previously reported sterile taxa were also found to be fertile, including 'Aphrodite', 'Diana', 'Helene' and 'Minerva'. Two hexaploids, 'Pink Giant' and Raspberry Smoothie™, were found to have reduced female fertility compared to tetraploids. Fertility testcrosses of pentaploid seedlings revealed a reduction in fertility compared to controls. The reduction in fertility of

pentaploids will likely lead to new, near sterile cultivars for the nursery industry. The combination of double flowers with pentaploid cytotypes will likely lead to completely sterile cultivars of althea.

Although floral traits are most important for breeders of althea, little is known about their segregation patterns. The objectives of this study were to determine segregation patterns in eyespot presence, flower color, and flower form. Over four years, thousands of flowering seedlings were observed representing F_1, F_2, and backcross families. Based on our results, we propose that eyespot presence is controlled by a single locus and that a recessive allele called *spotless* results in a complete elimination of color. The gene controlling *spotless* is likely located upstream in the flavonoid biosynthetic pathway. We also propose that flowers with white to blush-pink petal body color and a red eyespot are controlled by a single recessive allele called *geisha*. This trait exhibits incomplete dominance and is under epistatic control by *spotless*. It is likely located downstream in the delphinidin biosynthetic pathway, responsible for lavender, dark pink, and blue pigments. In addition to color segregation, depth of color irrespective of hue (CIE L*) was also investigated (*spotless* and *geisha* seedlings removed). The deepest pigments were measured in crosses among hexaploid 'Pink Giant', taxa homozygous dominant for *geisha*, and taxa heterozygous for *geisha*. Conversely, the lightest pigments were observed in crosses between taxa homozygous recessive for *geisha* and taxa heterozygous for *geisha*. Future efforts at eliminating the *geisha* allele from a breeding population may allow for quantitative improvement in total anthocyanin production. Observations on petal number inheritance revealed that seedlings produced a continuous distribution of petal numbers between the petal numbers of the two parents, with occasional transgressive segregants. The highest average petal numbers were found in seedlings resulting from the cross of double-flowered taxa. Flower size (petal area), varied significantly among cross combinations and flower forms. The largest petals were observed in the seedlings of single-flowered by double-flowered crosses. Concomitant upregulation or expression of genes controlling laminar growth in stamen may not only result in petaloid stamen, but may also result in increased laminar growth in the true petals, resulting in wider,

overlapping petals. However, further work must be undertaken to eliminate environmental effects on flower size estimates.

Breeding and Genetics of Lilacs and Hardy Hibiscus

by

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APPROVED:

Major Professor, representing Horticulture

Head of the Department of Horticulture

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Jason D. Lattier, Author

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CONTRIBUTION OF AUTHORS

Dr. Ryan Contreras was involved in the planning, design, analysis and interpretation of data in all chapters of the dissertation. For Chapter 3, Tyler Hoskins contributed to the interpretation of data and will continue taking data over subsequent years until flowering to confirm a reduction in juvenily in green seed treatments of lilac. For Chapter 6, Hsuan Chen developed the FISH protocol and guided the FISH analysis on the ploidy series and pentaploid population of *Hibiscus syriacus*. Hsuan Chen also helped develop the method for root tip cytology presented in the Appendix.

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CHAPTER 1: GENERAL INTRODUCTION

Nursery Industry

While the primary goal of agriculture is to produce plants that nourish the body, ornamental plants serve to feed mankind in a different, yet equally important way. Ornamental plants are a feast for the senses. Among their many uses, ornamental plants fill our landscapes with color, clean and perfume our air, and bring wildlife into our gardens. The ornamental nursery industry does not just sell plants; they sell products that improve the psychological well-being of society. The value and importance of aesthetics has built the vibrant United States nursery industry into a multi-billion-dollar industry (USDA, 2016). Some of the staples of this industry are the deciduous shrubs. These plants include spring- and summer-flowering, tough-as-nails taxa, including lilacs (*Syringa*) and althea (*Hibiscus syriacus*), which can be grown in a wide range of hardiness zones across the country. In a recent USDA Census on Horticulture Specialties, deciduous shrubs account for nearly 100 million units sold at a value of over $676 million in total sales (USDA, 2016). Nationwide, lilacs account for nearly 2 million units sold at a value of over $20 million in total sales, while hibiscus account for nearly 4.5 million units sold at a value of over $30 million in total sales (USDA, 2016).

In Oregon, greenhouse and nursery products combined made up the top agricultural commodity in 2016 at $909 million based on production value (ODA, 2017). The Oregon nursery industry had previously topped the billion-dollar mark in 2007 prior to the recession of 2008 (ODA, 2017). Since 2008, the value of nursery

crops in Oregon, and nationwide, has been steadily climbing and shows no sign of slowing down (ODA, 2017). Lilacs and hibiscus make up 0.275% of the Oregon nursery industries sales. In the last USDA Census on Horticulture Specialties, lilacs accounted for over 220,000 units sold at a value of over $2 million in total sales in Oregon (USDA, 2016). Hibiscus accounted for over 56,000 units sold at a value of over $500,000 in total sales in Oregon (USDA, 2016). Like many industries, the nursery industry thrives on of new and novel product releases. While engineers and designers create the next must-have phone, computer, or car, creating new ornamental plants requires the modern toolbox of plant breeding and genetics to reimagine what is possible for classic garden plants.

Lilacs

The fragrance of lilacs permeates the storied history of horticulture. Species scattered from the Balkan-Carpathian region of Europe to the farthest reaches of Asia have graced the halls of power and the homes of peasants alike. Lilacs are members of the Olive family, characterized by two-merous flowers, two anthers, two-loculed ovaries, and other reproductive structures. Phylogenetic analyses partitioned the lilac genus (*Syringa* L.) into six series: *Pubescentes*, *Villosae*, *Ligustrina*, *Ligustrae*, *Pinnatifoliae*, and *Syringa* (Li et al., 2012). Although success has been limited in creating interseries hybrids, interspecific and intraspecific hybridization throughout the centuries has produced a wide range of traditional and modern cultivars in the shrub lilacs. Within the shrub lilacs, most improvements and cultivar releases have come from series *Syringa*, *Pubescentes*, and *Villosae*. The breeding program at

Oregon State Univeristy (OSU) has focused on discovering the variation in cross-compatibility among taxa in these groups. We have also focused on cultivar development in two vastly different series, *Syringa* and *Pubescentes*.

While the bulk of species hail from the Orient, the common lilac, *Syringa vulgaris*, makes its native home in the embattled lands of the Balkans. The classic lilac's native range cuts westward from the Black Sea through the limestone mountain cliffs of Bulgaria and Romania down to the rocky edges of the Danube (Fiala and Vrugtman, 2008). This lilac took a circuitous route to the gardens of the West. *Syringa vulgaris* first travelled east to intoxicate the gardens of Istanbul before enthralling gardens of Vienna in the 1500s (Fiala and Vrugtman, 2008; Verdoorn, 1944). Needless to say, the lilac took Western Europe by storm. As the Dutch, French, and English gazed across the Atlantic with colonial aspirations, the prized lilac was pruned and packaged for its long journey to the New World. Here, gardeners and breeders would chart a new history for the common lilac (Fiala and Vrugtman, 2008). A world away, in the valleys of China's Loess Plateau near the Great Wall, the early blooming lilac was discovered—*Syringa oblata*, the only other member of section *Syringa*. These distant cousins of the common lilac were first discovered throughout vast, temperate forest floors of Hebei and Shandong provinces. The reunion of these two far-flung flowers would create magic in gardens of early lilac hybridizers (Fiala and Vrugtman, 2008).

Breeding lilacs was rare prior to the late 1800s. Most cultivars arose from selected seedlings planted by nurserymen and the occasional botanical aficionado. Early selections focused on improving form, flower colors, and the coppery flush of

spring leaves in common garden lilac (Fiala and Vrugtman, 2008). French nurseryman Victor Lemoine, a rarity in his time, became a student of genetics and transitioned from selector to hybridizer. Lemoine's genius was to painstakingly pollinate tiny, deformed pistils of little known, double-flowered cultivars with pollen from single-flowered selections of *Syringa vulgaris* and *Syringa oblata* (Fiala and Vrugtman, 2008). This resulted in a breeder's dream collection of giant, double-flowered lilacs in varied colors, forms, and bloom times, many of which were the new interspecific hybrid *Syringa* ×*hyacinthiflora* (Lemoine, 1878). Much of the boom in lilac cultivars during the 1900s can be attributed to this family of breeders and the 214 cultivars they released (Fiala and Vrugtman, 2008; Hirtz, 1993).

In North America, several true hybridizers emerged to have a lasting impact on lilac breeding. Hulda Klager's life was spent as a true pioneer—travelling from her native home of Germany in 1864 to Washington State by her teenage years (Fiala and Vrugtman, 2008). In her spare time, she became an avid student of botany and corresponded with the great hybridizer Luther Burbank. Over forty inspired years of breeding with her "Magic Three" cultivars, she created a panoply of exquisite, disease-resistant, colorful hybrids of lilacs (Fiala and Vrugtman, 2008). Though she named 100 new cultivars, only 13 cultivars were released commercially before a devastating flood destroyed her garden in 1948. In response, a community of friends, gardeners, and customers helped rebuild the garden—a public and National Historic Site in the small town of Woodland, Washington (Collins, 1948; Fiala and Vrugtman, 2008).

In the early 1900s, the Lemoines received a visit from New York gardener T.A. Havemeyer. After an inspirational trip to France, he returned home with bundles of plants and a mission to breed more colorful, large, single-flowered lilacs. There is no record of his crosses, but Havemeyer was unique in his approach of building an extensive breeding collection of the finest specimens from France and North America (Fiala and Vrugtman, 2008). His 42-acre estate on Long Island produced 45 new cultivars of improved lilacs. Mark Eaton maintained the collection upon Havemeyer's death and would see eleven more cultivars produced from the collection (Fiala and Vrugtman, 2008; Wister, 1953).

Southern California must have seemed like a strange place to find a lilac breeder in the 1950s. However, Walter Lammerts at Descanso Gardens was busy selecting forms of *Syringa ×hyacinthiflora* for bloom in mild climates (Fiala and Vrugtman, 2008). Most lilac experts understood the importance of long winters of chilling temperatures to break flower bud dormancy. However, his cultivar 'Lavender Lady' ignored all the rules and brought the fragrance of lilac south. Descanso would go on to release 18 cultivars with low chilling requirements (Fiala and Vrugtman, 2008). While Lammerts and Sobeck of Descanso focused on mild climate lilacs, the legendary Frank Skinner was busy selecting for improved cold hardiness in frigid Manitoba within USDA hardiness zone 2b (Fiala and Vrugtman, 2008). Wild *Syringa oblata* (Korea) given to him by Arnold Arboretum proved a unique parent that produced 20 of the best cultivars of *Syringa ×hyacinthiflora* for cold weather gardens (Fiala and Vrugtman, 2008).

Occasionally, hybridizers become infatuated with mutations that others overlook. A funny thing happened in Rochester, NY when director of parks Alvan Grant collected seeds of the large, double-flowered Lemoine introduction 'Edith Cavell' (Fiala and Vrugtman, 2008). One plant was not double, but was a multi-petaled, single white with waxy petals, dubbed the "primrose" form. What's more—it was smaller, slow-growing with thick leaves (Fiala and Vrugtman, 2008). This specimen was named 'Rochester' and Richard Fenicchia of Highland Botanical Park recognized how special a parent this would be (Millham, 2004). Using 'Rochester' as a seed parent, Fenicchia hybridized it with other elite cultivars producing new compact, colorful, large-flowered lilacs known as the Rochester Strain (Fiala and Vrugtman, 2008; Millham, 2004).

In Ohio, a humble parish priest, professor, and plant enthusiast, John Fiala, was busy building one of the great modern lilac collections. Over many decades, Fiala improved cultivars of *Syringa vulgaris* and *Syringa* ×*hyacinthiflora*, and experimented with wide hybridizations among rare species (Fiala and Vrugtman, 2008). Fiala also experimented with colchicine, a chemical that doubles chromosome numbers in plants (polyploidy) and induces novel, ornamental mutations. Many of Fiala's named cultivars resulted from his colchicine experiments, yet few have been confirmed as polyploids using modern tools (Fiala and Vrugtman, 2008). His gardens at Falconskeape produced 50 named cultivars. His research and breeding notes were documented in his extensive publications, providing reference material for all future publications on lilacs, including this introduction.

Don Egolf of the US National Arboretum focused on combining two previously-mentioned traits—disease resistance and low chill. Egolf determined the best way to begin such a breeding program was to survey thousands of marketed cultivars of *Syringa vulgaris* and *Syringa* ×*hyacinthiflora* and assess their breeding potential and susceptibility to powdery mildew (Fiala and Vrugtman, 2008). Egolf sought to extend the southern limits of growing lilacs through selective hybridization. The posthumous release of three cultivars, 'Betsy Ross', 'Declaration', and 'Old Glory' provided new possibilities for southern low chill lilacs free from powdery mildew (Fiala and Vrugtman, 2008).

Far from the European home of the common lilac, the first member of series *Pubescentes* was discovered in China. *Syringa pubescens* was found growing in areas of open canopy among spruce, oak, and linden-birch forests. Populations were also found thriving in the mountains at moist, high-elevation altitudes (Fiala and Vrugtman, 2008). Most Chinese species remained undiscovered until the late 1800s. As China opened up to missionaries, botanists, and plant explorers, the family of lilacs grew rapidly during the 1800s and early 1900s. As plant explorers turned their gaze to the mountains of Korea in the 1900s, not only were new forms of *Syringa oblata* discovered, but the *Pubescentes* were further expanded. During one of these expeditions, a selection from seed collected by Elwyn Meader in the Pouk Han Mountains would go on to become the ever-popular 'Miss Kim' lilac (Fiala and Vrugtman, 2008).

While stationed in China as a translator, the Belgian Joseph Hers began a storied correspondence with the Arnold Arboretum in his avocation as a plant hunter.

Hers was the first to note remontant (repeat) flowering in a wild specimen of *Syringa pubescens* (Fiala and Vrugtman, 2008). Material was later evaluated at the Arnold Arboretum where director Sargent commented, "…if it keeps up its habit of flowering a second time in autumn it will at least be interesting even if other lilacs are more beautiful." This understated observation stands in stark contrast to the ever-increasing interest of modern horticulture in developing remontant shrubs.

In contrast to the long history of breeding in common lilac, and the thousands of named cultivars it produced, breeding efforts in series *Pubescentes* have been rare and limited to only the most adventurous lilac hybridizers. Frank Skinner was improving cold-hardiness in a range of trees and shrubs at his nursery in Manitoba which included not only common lilac, but also lesser known lilac species (Fiala and Vrugtman, 2008). He was a contemporary of the intrepid Isabella Preston and began producing his own improvements on *Syringa* ×*prestoniae* hybrids. However, it was from E.H. Wilson's Diamond Mountain expedition in Korea that Skinner received seed of *Syringa pubescens* subsp. *patula*, which he began hybridizing with selections of *Pubescentes* from China. During the course of his life, Skinner released 144 new cultivars of ornamental plants, including many improved and wide hybrids in lilacs (Fiala and Vrugtman, 2008). In Ohio, the devout plantsman and consummate lilac collector Father Fiala delved into the world of series *Pubescentes*, creating hybrids with the little-known, spicy fragranced *S. pubescens* subsp. *julianae*.

Most of the great advances in breeding within the *Pubescentes* have occurred in the modern era. In the mid-1900s, the team of plant hunters and breeders at the University of New Hampshire, including Elwyn Meader and Albert Yeager, were

responsible for some of the early advances in selection and breeding of *Pubescentes*—including the stunning 'Miss Kim' lilac (Fiala and Vrugtman, 2008). By the 1960s, the French academic Georges Morel produced a complex cross that would later become 'Josée' and was introduced into the trade by Pépinières Minier. Further groundbreaking work was contributed by Neal Holland at his nursery in North Dakota which produced the Fairytale® series of lilacs introduced by Bailey Nurseries (Fiala and Vrugtman, 2008). These crosses utilized the special *Syringa meyeri* 'Palabin' discovered high in the mountains of Korea and noted for its ability to set abundant seed. Canadian horticulturists Frank and Sara Moro (Select Plus International Lilac Nursery, Quebec) have also introduced improved cultivars of *Pubescentes* including 'Cinderella', 'Colby's Wishing Star', and 'Snowstorm' (Fiala and Vrugtman, 2008). Other industry-leading *Pubescentes* have come from the Proven Winners® remontant series, including the powerhouse Bloomerang® series of lilacs.

The breeding program at OSU has used both traditional and modern breeding techniques to improve taxa in sections *Syringa* and *Pubescentes*. In section *Syringa*, we have focused on producing compact, disease-resistant, floriferous selections through controlled crosses of elite cultivars. Crosses have been designed to evaluate heritability of traits such as disease resistance, low chill, foliar pigment, flower form and flower color. The Willamette Valley provides high disease pressure for two ailments that perpetually plague the lilac enthusiasts—bacterial blight and powdery mildew. Bacterial blight can be devastating to lilacs flushing in the cool, wet spring of the Pacific Northwest (Fiala and Vrugtman, 2008). While not as damaging as

bacterial blight, powdery mildew's frustrating coat of cottony mycelium can turn a lilac into an eyesore. Breeders continue to probe the genetic diversity within lilacs for weapons to combat these common diseases, and toward that goal, we are currently evaluating progeny from hundreds of crosses.

Despite Fiala's call for cytological work in colchicine-treated lilacs, a gap still exists in the scientific literature and a comprehensive study is long overdue. The Ornamental Plant Breeding Program at OSU has used flow cytometry to screen our collection of parents and a subset of our progeny. Screening parents allows us to determine the ploidy variation available to breeders, and screening progeny identifies parents that produce unreduced gametes (pollen or eggs). Parents exhibiting ploidy variation in their gametes produce progeny with ploidy variation—increasing the likelihood of novel phenotypes available to the breeder.

The ornamental breeding program at OSU is also exploring new possibilities in lilac breeding hidden within series *Pubescentes*. Topping the list of important breeding objectives is the development of remontant, disease-free cultivars. In the past several years, after making numerous crosses, we have observed a high degree of variability among our parents and progeny populations in susceptibility to bacterial blight and degree of remontancy. In response, we are using next-generation sequencing to discover genetic markers for remontancy and disease resistance. Identifying these markers will allow our program and future breeders to include marker-assisted selection in their breeding toolbox. Genetic markers will enable identification of progeny with genes for disease resistance and/or remontancy at the seedling stage. This will reduce the size of populations that need to be grown. Even

though traits such as reblooming were identified decades ago, we are still in the process of realizing the full potential of this exciting group of plants. New technology, while keeping an eye to the groundbreaking work of our predecessors, is allowing us to make what's old new again.

Our breeding program continues the journey toward new cultivars of lilac that fit modern gardening trends. There are still improvements to be made for modern landscapes by increasing disease resistance, enhancing flower and foliage colors, and improving growth habit. We are in the enviable position of having access to our predecessors' work while also having modern tools such as flow cytometry and molecular markers. This dissertation presents the results of experiments on lilac involving cross-compatibility, fertility tests, wide hybridization, embryo rescue, flow cytometry, cytology, genotyping-by-sequencing, linkage analyses, and construction of genetic linkage maps.

Althea

Althea (*Hibiscus syriacus*), also known as Rose of Sharon, represents one of a handful of species in this diverse genus to extend its natural range into temperate climates, along with *H. paramutabilis* and *H. sinosyriacus* (Bates, 1965). Western botanists first became aware of althea in the gardens of Syria, which led Linneaus to refer to it as "Syrian" and from which it gets its specific epithet (Lawton, 2004; Walker, 1999). However, althea originated in eastern Asia and its cultivation has been documented in early Chinese paintings and literature (Lawton, 2004). Popularity of *H. syriacus* remains undiminished in South Korea where it is the

national flower and known as “mugung-hwa,” meaning “endless” (Lawton, 2004). Although this name can have a terrifying connotation to a Ph.D. student working within the taxa, the name references the blooming ability of mature plants, often producing more than 3,000 flowers in a season (Lawton, 2004).

Althea has a centuries-long history in the American nursery industry. Althea has graced American gardens since colonial times with its large, tropical blooms and bright summer colors. What sets althea apart from its equally desirable cousins, such as *H. rosa-sinensis*, is its hardy above-ground stems and the color blue (Walker, 1999). True blue flowers are rare among the species of *Hibiscus*, and even rare within althea, with only a few popular cultivars. Most flowers fall in the range of soft, anthocyanin pigments including blush pink, pink, lavender, and blue, and are usually offset by a brilliant red eyespot (Walker, 1999). Despite its history and garden merits, althea provides fertile ground for modern plant breeders, as noted by Dr. Michael Dirr (2009), “*Hibiscus syriacus* is a gold mine for a breeder because of the potential for myriad flower forms quickly and fully expressing their attributes.”

Previous breeders have focused on floral traits such as flower color, flower size, and number of petaloid stamen. Based on number of petals, cultivars have previously been classified as single-flowered (five true petals), semi-double (five true petals + some petaloid stamens), and full double (five true petals + all petaloid stamens) (Contreras and Lattier, 2014). Breeders have also released cultivars with novel forms such as dwarfs (e.g. Lil’ Kim™) and cultivars with novel variegated foliage (e.g. Sugar Tip®). For such an important and historic species with a relatively short generation time compared to most woody shrubs, little is known about the

inheritance of floral traits in althea. Flower colors and forms are diverse. For instance, most taxa produce flowers with red eyespots, yet several white-flowered taxa completely lack eyespots (e.g. 'Diana', White Chiffon®, and 'Buddha Belly'). Most flowers with eyespots also produce pigments in the petal body, such as dark pink, lavender and blue. Yet, some flowers produce eyespots with petal bodies that lack pigment entirely (e.g. 'Helene' and Lil' Kim™). Heritability studies of flower color and flower form are long overdue, and the results could greatly benefit althea breeders in the years to come.

Another major goal of althea breeders has been to develop sterile cultivars. Flowers of most althea cultivars are fertile, and fertilized flowers produce capsules containing dozens of seed. Don Egolf, former breeder at the U.S. National Arboretum, used a technique of polyploid-induction to create sterile or near-sterile cultivars. To achieve this, Egolf used colchicine, a spindle-fiber inhibitor, to double the chromosome number of the elite taxon, 'William R. Smith'. Hybrids between the induced polyploid and advanced selections resulted in several cultivars ('Diana', 'Helene', 'Minerva', and 'Aphrodite'), which are widely available in the nursery industry (Egolf, 1970, 1981, 1986, 1988). Egolf based his theory on reports that althea existed primarily as a diploid, and his interploid hybrids were sterile triploids. However, previous reports as well as a recently published draft genome (Kim et al., 2017) confirm that *H. syriacus* exists primarily as a tetraploid. Therefore, it is possible that many of the colchicine-induced polyploids were octaploid and their resulting progeny were hexaploids with reduced fertility. However, since no comprehensive genome size and ploidy survey of current cultivars exists, breeders

lack information critical to using elite polyploid cultivars in novel cross combinations.

Polyploidy in *H. syriacus* may complicate segregation analysis for important traits. Autopolyploids often have complex segregation patterns, even for single gene traits, yet allopolyploids (amphidiploids) can have segregation ratios that follow simple diploid Mendelian inheritance patterns. Previous work in cousins of *H. syriacus*, in *Hibiscus* section *Furcaria*, revealed that all species were allopolyploid in origin, with tetraploids, hexaploids, octaploids, and decaploids discovered (Menzel and Wilson, 1969; Wilson, 1994, 1999). In addition, induced autopolyploids in *H. acetocella* proved to be completely sterile, possibly due to the production of multivalents (Contreras et al., 2009). Therefore, it remains to be seen if *H. syriacus* can tolerate high level polyploidy resulting from induced autopolyploidy without yielding sterile plants. However, Egolf's success with his colchicine-induce 'William R. Smith' lends evidence to the success of polyploid induction and interploid hybridization in althea.

In addition to ploidy levels, little is known about the pedigrees of many of the elite cultivars available in the nursery industry. Breeding programs can be hampered by not having pedigree information on their parent taxa. Often, self-incompatibility and specific cross-incompatibilities can exist among selections in a breeding population limiting seed production. In crosses that do produce seed, inbreeding depression can limit advancement if numerous cross-compatible combinations are not identified when building progeny populations. Therefore, cross-compatibility and fertility studies among elite cultivars could aid future althea breeders.

This dissertation presents the results of experiments on *H. syriacus* involving segregation analyses, fertility tests, wide hybridization, cross-compatibility, creation of a ploidy series, and fluorescent in situ hybridization.

Literature Cited

Collins, D. 1948. A lifetime with lilacs. Flower Grower 35: 830.

Contreras, R. and J.D. Lattier. 2014. Targeted breeding of rose-of-sharon to improve a garden classic. Digger (August 2014):47-50.

Contreras, R.N., J.M. Ruter, and W.W. Hanna. 2009. An oryzalin-induced autoallooctaploid of *Hibiscus acetosella* 'Panama Red". J. Amer. Soc. Hort. Sci. 134:553-559.

Dirr, M.A. 2009. Manual of woody landscape plants: their identification, ornamental characteristics, culture, propagation, and uses. 6th Ed. Stipes Publishing, Champaign, Illinois.

Egolf, D.R. 1970. *Hibiscus syriacus* 'Diana', a new cultivar. Baileya. 17:75-78.

Egolf, D.R. 1981. 'Helene' rose of sharon (althea). HortScience 16:226-227.

Egolf, D.R. 1986. 'Minerva' rose of sharon (althea). HortScience 21:1463-1464.

Egolf, D.R. 1988. 'Aphrodite' rose of sharon (althea). HortScience 23:223.

Fiala, J.L. and F. Vrugtman. 2008. Lilac: a gardener's encyclopedia. 2nd ed. Timber Press, Portland, OR.

Hirtz, F. 1993. Victor Lemoine, un grand nom de l'horticulture mondiale. Thesis, Diplôme d'Etat de Docteur en Pharmacie, Université de Nancy.

Kim, Y.-M., S. Kim, N. Koo, A.-Y. Shin, S.-I. Yeom, E. Seo, S.-J. Park, W.-H. Kang, M.-S. Kim, J. Park, I. Jang, P.-G. Kim, I. Byeon, M.-S. Kim, J. Choi, G. Ko, J. Hwang, T.-J. Yang, S.-B. Choi, J.M. Lee, K.-B. Lim, J. Lee, I.-Y. Choi, B.-S. Park, S.-Y. Kwon, D. Choi, and R.W. Kim. 2017. Genome analysis of

Hibiscus syriacus insights of polyploidization and indeterminate flowering in woody plants. DNA Res. 24:71-80.

Lawton, B.P. 2004. *Hibiscus* – hardy and tropical plants for the garden. Timber Press, Portland, OR.

Lemoine, V. 1878. *Syringa hyachinthiflora plena*. Catalogue 78, p.6. Nancy, France.

Li, J., B. Goldman-Huertas, J. DeYoug and J. Alexander, III. 2012. Phylogenetics and diversification of *Syringa* inferred from nuclear and plastid DNA sequences. Castanea 77: 82-88.

Menzel, M.Y. and E.D. Wilson. 1969. Genetic relationships in *Hibiscus* sect. *Furcaria*. Brittonia 21:91-125.

Millham, K. 2004. Richard Fenicchia: great plantsman and hybridizer. Lilacs 33: 111-113.

Oregon Department of Agriculture (ODA). 2017. Nursery back on top as Oregon's #1 ag commodity. Accessed: 5 August 2017. http://odanews.wpengine.com/nursery-back-on-top-as-oregons-1-ag-commodity/

U.S. Department of Agriculture (USDA). 2016. Census of horticultural specialties for 2014. Accessed: 5 August 2017. https://www.agcensus.usda.gov/Publications/2012/Online_Resources/Census_of_Horticulture_Specialties/

Verdoorn, Frans, editor. 1944. Ghiselin de Busbecq (Busbequius), Ogier. In: On the aims and methods of biological history and biography with some notes for the collaborators of the Index Botanicorum. Chronica Botanica 8:441-444.

Walker, J. 1999. *Hibiscus*. Cassel, London, England.

Wilson, E.D. 1994. The genome biogeography of *Hibiscus* L. section *Furcaria* DC. Genet. Res. Crop Evol. 41:13-25.

Wilson, E.D. 1999. Revision of *Hibiscus* section *Furcaria* (Malvaceae) in Africa and Asia. Bul. Natl. History Museum London Bot. 29:47-79.

Wister, J.C. 1953. Lilacs for America: Report of the 1953 lilac survey committee of the American association of botanical gardens and arboretums. Swarthmore, PA: Arthur Hoyt Scott Horticultural Foundation.

CHAPTER 2: INTRASPECIFIC, INTERSPECIFIC, AND INTERSERIES CROSS-COMPATIBILITY IN LILAC

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CHAPTER 2: INTRASPECIFIC, INTERSPECIFIC, AND INTERSERIES CROSS-COMPATIBILITY IN LILAC.

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Abstract. Lilacs are a group of ornamental trees and shrubs in the Oleaceae family consisting of 22 to 30 species from two distinct centers of diversity: the highlands of East Asia and the Balkan-Carpathian region of Europe. There are six series within genus *Syringa*: *Pubescentes*, *Villosae*, *Ligustrae*, *Ligustrina*, *Pinnatifoliae*, and *Syringa*. Intraspecific and interspecific hybridization are proven methods for developing lilac cultivars with improved flowering, new foliar phenotypes, and improved growth habits. However, reports of interseries hybridization are rare and limited to crosses between taxa in series *Syringa* and *S. pinnatifolia* in series *Pinnatifoliae*. Though hundreds of improved lilac cultivars have been introduced, fertility and cross-compatibility among cultivars, species, and series have yet to be formally investigated. Over three years, a cross-compatibility study was performed using elite cultivars and species of shrub-form lilacs in series *Syringa*, *Pubescentes*, and *Villosae*. A total of 114 combinations were performed at an average of 243 ± 27 flowers pollinated per combination. For each combination, we recorded the number of inflorescences and flowers pollinated, number of capsules, number of seeds, number of seedlings germinated, and number of albino seedlings. Fruits and seeds were produced from interseries crosses, but no viable seedlings were

recovered. A total of 2,177 viable seedlings were recovered from interspecific and intraspecific combinations in series *Syringa*, *Pubescentes,* and *Villosae*. Albino progeny were produced only from crosses with *S. pubescens* subsp. *patula* 'Miss Kim'. In vitro germination was attempted on 161 abortive interseries seed and resulted in only three in vitro germinations from hybrids of *S. pubescens* Bloomerang® x *S. vulgaris* 'Ludwig Spaeth'. None of the seedlings survived, yet cotyledons produced excessive callus providing material for future efforts to induce embryogenic shoots. This study is a comprehensive investigation of lilac hybridization, and the knowledge gained on cross compatibility relationships will aid future efforts in lilac cultivar development.

Introduction

Syringa L. is a diverse genus in the olive family (Oleaceae) consisting of 22 to 30 species from two distinct centers of diversity: the highlands of East Asia and the Balkan-Carpathian region of Europe (Kochieva et al., 2004). The majority of lilacs are native to the Asian center of diversity with only *S. vulgaris* and *S. josikaea* native to southeastern Europe (Kim and Jansen, 1998). Hundreds of lilac cultivars have been developed as ornamentals and are ubiquitous in temperate gardens around the world. Historically, the most popular cultivars of lilacs originated from the European species *S. vulgaris,* primarily grown for its fleeting spring blooms of purple, pink, blue, or white fragrant flowers. Previous phylogenies have divided the species into subgenera and four series (Rehder, 1945), which were later confirmed as monophyletic groups using plastid DNA (Kim and Jansen, 1998). The current

phylogeny of the genus by Li et al. (2012) based on nuclear and plastid DNA sequences recognizes six series within *Syringa*: *Pubescentes*, *Villosae*, *Ligustrina*, *Ligustrae*, *Pinnatifoliae*, and *Syringa* (=*Vulagares*).

Each series has distinguishing morphological features. Series *Syringa* is unique by having simple, glabrous leaves while series *Pubescentes* has pubescent leaves (Li et al., 2012). Series *Villosae* is distinct by having inflorescences develop from a single terminal bud with lateral buds becoming vegetative shoots (Kim and Jansen, 1998). *Ligustrina* differs from other lilacs by its privet-like flowers (short, white corolla tubes with long exerted anthers) and growth habit as a large tree (Kim and Jansen, 1998). *Pinnatifoliae* is distinguished by having pinnately compound leaves (Li et al., 2012). *Ligustrae* contains several privets (*Ligustrum spp.*) nested within the lilacs (Li et al., 2012).

Lilacs are of major economic importance in the U.S. nursery industry. In 2014, total sales nationwide topped 1.8 million generating over $20 million in total revenues (USDA, 2016). Intraspecific and interspecific hybridization have proven to be valuable methods for development of elite lilac cultivars. Interspecific hybridization has been particularly useful at producing cultivars with improved flowering and new foliar phenotypes (Table 1). Lilac breeding was scarce prior to the 1800s, a time when selections focused on improved form, flower color, or spring flush in chance seedlings (Fiala and Vrugtman, 2008). Early advancements in lilac breeding produced highly vigorous interspecific hybrids including *S.* ×*hyacinthiflora* from crosses between *S. oblata* and *S. vulgaris* by the Lemoine nursery (Lemoine, 1878; Sax, 1930). This nursery was responsible for 214 cultivars and a spike in

popularity of lilacs in the 1900s (Fiala and Vrugtman, 2008; Hirtz, 1993). Many breeders emerged to produce cultivars with a wide range of ornamental traits. Descanso Gardens in Southern California and the US National Arboretum focused on improving *S.* ×*hyacinthiflora* hybrids for southern climates using traits by incorporating low chilling requirements and powdery mildew resistance (Fiala and Vrugtman, 2008).

Cultivar improvement in the series *Villosae* began its ascendancy with complex interspecific hybridization involving *S. reflexa* by Isabella Preston at the Central Experimental Farm in Ottawa, Canada (Fiala and Vrugtman, 2008). A total of 47 cultivars were introduced from the interspecific hybrids *S.* ×*prestoniae* and *S.* ×*josiflexa* which were created by crossing several species in series *Villosae* (*S. villosa*, *S. reflexa*, and *S. josikaea*) (Table 1) (Fiala and Vrugtman, 2008). One of Preston's contemporaries in Canada, Frank Skinner, also produced similar interspecific hybrids in *Villosae*, several of which are still available in the trade (Fiala and Vrugtman, 2008).

Ornamental traits in series *Pubescentes* have been noted since the early 1900s when the director of the Arnold Arboretum, Charles Sargent, noted in a wild-collected specimen of *Syringa pubescens*, "…if it keeps up its habit of flowering a second time in autumn, it will at least be interesting even if other lilacs are more beautiful." Remontancy (or reblooming) as noted by Sargent would become one of the most pursued traits by modern lilac breeders (Fiala and Vrugtman, 2008). Early cultivar introductions in series *Pubescentes* exhibited improved form and flowers in addition to cold hardiness from wild-collected *S. pubescens* subsp. *patula* from EH

Wilson's Diamond Mountain expedition in Korea (Fiala and Vrugtman, 2008). Most new cultivars in series *Pubescentes* are prolific flowering, compact, disease resistant lilacs with several cultivars exhibiting summer remontancy, such as *S. pubescens* Bloomerang® Purple.

In contrast to the success of interspecific hybridization, interseries hybridization has proven more difficult with the only successful hybrids from crosses between taxa in series *Syringa* (*S. oblata* var. *giraldii*, *S. vulgaris*, *S. laciniata*, and *S.* ×*hyacinthiflora*) with *S. pinnatifolia* in series *Pinnatifoliae* (Pringle, 1981). Interseries hybridization has been a goal of lilac breeders for nearly a century, as illustrated by early reports on lilacs: "…combinations of the early blooming *Syringa vulgaris* varieties with the late *Villosae* species would undoubtedly be of value if they could be made…" (Sax, 1930). Previous attempts to create interseries hybrids resulted in highly abortive fruits with no germination of recovered seeds (Pringle, 1981).

Abortive seeds in lilacs have been explored in previous research. Anatomical studies on *S. villosa* Vahl, an Asiatic lilac with naturally high rates of seed abortion, found that after cross-pollination, embryos developed normally through the globular, heart-shaped, torpedo, and cotyledon stages before the embryo and endosperm began to degrade (Chen et al., 2012). Few embryo rescue studies have been attempted in lilacs. However, Zhou et al. (2003) successfully cultured immature embryos on Monnier's Medium (1990) supplemented with 1-naphthaleneacetic acid (NAA), 6-benzylaminopurine (BAP), glutamine (Gln), and a high concentration of sucrose, indicating that tissue culture may be a platform for rescuing abortive seeds of lilac

hybrids. Even if in vitro germination fails, callus developed from hybrid tissue may provide another source material for producing interseries hybrids. Lilac somatic embryogenesis protocols using cotyledons have recently been developed for *S. reticulata* var. *mandshurica* (Liu, 2013).

Though hundreds of improved lilac cultivars have been introduced, fertility and cross-compatibility between cultivars, species, and series have yet to be investigated in a formal study. The objectives of this study were to 1) investigate cross-compatibility of elite cultivars of lilac in intraspecific, interspecific, and interseries combinations and 2) investigate the potential for interseries hybridization and in vitro embryo rescue of abortive embryos.

Materials and Methods

Parent Material. Parent plants were collected from nurseries, gardens, and arboreta from 2009 to 2014 (Table 2) who provided cultivar and trademark names. Taxonomic designations reflect current phylogenies and revisions, including the use of subspecies designations in *Pubescentes* (Chen et al., 2009). Representative species and cultivars were obtained from series *Syringa, Pubescentes*, and *Villosae* focusing on elite cultivars improved for one or more important horticultural traits including novel flower colors and forms, novel leaf pigments, and novel forms including dwarf habits.

Flower colors included white, pink, blue, and purple, with one cultivar, *S. vulgaris* ‘Sensation’, having picotee flowers (Fig. 2.1A) in which the petal edges exhibit darker or lighter pigment. Flower forms included single flowers (Fig. 2.1B)

and double flowers, with some exhibiting hose-in-hose double flowers (Fig. 2.1C). Double flowers in lilac often represent a case of neoheterotrophy where additional floral whorls are added to a perfect flower leading to supernumerary petals (Dadpour et al., 2011). Double flowers can also arise from mutations leading to petaloid sepals (Fiala, 2008). Both cases leave reproductive whorls intact and allow for double-flowered cultivars to be used in reciprocal crosses. Foliar pigments were rare across the breeding population, occurring in a spring flush of yellow or purple leaves (Fig. 2.2). *Syringa emodii* in series *Villosae* was the only taxon that produced a yellow flush of leaves (Fig. 2.2A). Purple color of spring foliage was limited to members of series *Syringa* with purple flowers, but was most pronounced in *S.* ×*hyacinthiflora* 'Old Glory' (Fig. 2.2C). Novel variations in form were limited to two dwarf cultivars in series *Syringa*, *S. vulgaris* Tiny Dancer™ and *S. vulgaris* 'Prairie Petite'.

Crosses. During the spring and summer of 2013, 2014, and 2015, a total of 27,645 cross pollinations were made among cultivars, species, and series. Of these, 114 crosses were performed with an average of 243 ± 27 flowers pollinated per cross. For each series, three types of crosses were attempted: intraspecific, interspecific, and interseries (Table 2.3, 2.4, and 2.5, respectively). Within each series, crosses made with at least one interspecific parent (e.g. *S.* ×*hyacinthiflora* x *S. oblata*) were classified as interspecific. The majority of elite cultivars in *Villosae* were interspecific hybrids; each replicate of an unimproved species was collected from a single source. For example, we received *S. emodii* from a single Index Seminum source (Hohenheim Gardens). Consequently, crosses with *Villosae* focused on interseries and interspecific crosses since intraspecific crosses would likely involve

significant inbreeding due to the limited number of sources, all of which were of garden origin.

Each year, fresh pollen was collected and stored in small petri dishes over desiccant (Drierite™; W.A. Hammond Drierite Co. Ltd., Xenia, OH) in a refrigerator at 4 ˚C (Fig. 2.3A). Two to four anthers from each flower were collected; no petal tissue was stored with the pollen. Prior to pollination, open flowers were removed on all inflorescences and saved in glassine bags for pollen collection and subsequent use in reciprocal crosses. Individual flowers were emasculated prior to anthesis and pollinations were made in a glasshouse kept free of pollinators with day/night temperatures of 25/20 °C and a 16-h photoperiod. Each flower was pollinated using a small paintbrush two to three times post-emasculation over consecutive days (Fig. 2.3B). All paintbrushes were sterilized before pollinations and between successive pollinations for all crosses using 70% ethanol. Incidences of self-pollination were tested by covering two inflorescences per plant with organza bags, and shaking occasionally to induce pollination. Self-pollination can also occur if pollen is released during emasculation. Self-pollination during the emasculation phase was tested by emasculating multiple inflorescences (200+ flowers) on two proven fertile parents (*S. vulgaris* ‘Angel White’ and *S. vulgaris* ‘Ludwig Spaeth’) and covering the inflorescences with organza bags. All inflorescences were labelled with jewelry tags on which the cross combination, date, and number of flowers pollinated were recorded. Additionally, all crosses were recorded in a field notebook. Developing fruits were counted throughout the summer and dry fruit were collected prior to dehiscence during fall (Fig. 2.3C). Data were collected on number of pollinated

inflorescences, number of pollinated flowers, and fruit set. During fall, seeds were cleaned and counted prior to cold stratification during winter.

Seed Germination. Seeds were placed in plastic bags filled with moist stratification media consisting of half perlite (Supreme Perlite Company, Portland, OR) and half Metro-Mix Professional Growing Mix (Sun Gro Horticulture, Agawam, MA). Seeds were cold-stratified for 10 weeks at 4 °C. After stratification, seeds were sown in 1.3-L containers filled with Metro-Mix Professional Growing Mix and treated once with Kocide® 2000 (DuPont™, Wilmington, DE) at 0.3 $mg \cdot L^{-1}$ (Fig. 2.3D). For each cross, lots of no more than 30 seeds were planted per pot. All seedlings were germinated in a glasshouse under the conditions described above. Data collected over the course of the winter included number of germinated seeds, number of albino seedlings, and number of viable, green seedlings. Individual seedlings were accessioned and potted during late winter into tree tube trays (0.22 L per cell) (Growers Solution; Cookeville, TN). Plants were moved into a lath structure to acclimate during spring (Fig. 2.3E). Acclimated plants were potted into 2.5-L containers filled with douglas-fir-based potting substrate during summer and grown in full sun under overhead irrigation.

In vitro germination. Capsules were observed to progress from pollination to dehiscence over a 20- to 30-week period in the glasshouse. In 2013, observations of early fruit abortion at six weeks post-pollination in the interseries cross *S. oblata* x *S. pubescens* Bloomerang® Purple prompted an attempt at embryo rescue of a subset of developing fruit (Table 2.6). In subsequent years, fruits were allowed to dehisce early, and seeds were sown according to the methods listed above. For in vitro

germination, interseries hybrid fruit from five crosses (Table 2.6) were collected 7 weeks after pollination. Green fruits were collected and immediately surface sterilized by rinsing in a 70% ethanol solution for 30 seconds followed by a soak in a 6.15% sodium hyperchlorite solution with several drops of the surfactant (Tween 20; Acros Organics™, Fair Lawn, NJ). Fruits were triple-rinsed and temporarily stored in filter-sterilized, autoclaved water. Fruits were dissected in a sterile, laminar flow hood using a dissecting microscope. Green seeds were removed from capsules into sterile petri dishes containing an aqueous solution of L-ascorbic acid at 25 $mg \cdot L^{-1}$ to reduce oxidation. Embryo extraction at this early stage proved too difficult and damaging to young tissues; intact dissected seeds were cultured on embryo rescue medium (Fig. 2.4A).

Dissected seeds were cultured on Monnier's medium according to the lilac embryo rescue protocol of Zhou et al. (2003) and incubated under standard culture conditions (24 ± 2 °C and a 16-h photoperiod of 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by cool-white fluorescent lamps). Single seeds were incubated on 10 mL of the embryo rescue medium in 150-mm culture tubes. Tubes were capped and sealed with Parafilm® (American National Can Co.; Menasha, WI). A total of 161 seeds were placed on germination medium, representing five interseries crosses. Culture tubes were completely randomized and maintained in racks of 40 tubes. Germination was observed and recorded over 3 months. Upon germination, seedlings and callus were transferred to shoot regeneration medium composed of MS basal salts and vitamins, 5 μM BAP (6-benzylaminopurine), 0.5μM IBA (indole-3-butyric acid), 100 $mg \cdot liter^{-1}$ *myo*-Inositol, 100 $mg \cdot liter^{-1}$ MES monohydrate [2-(N-Morpholino) ethanesulfonic

acid], and 30 $g \cdot L^{-1}$ sucrose. The solution containing basal salts, vitamins, and hormones (Phytotechnology Laboratories, Shawnee Mission, KS) was adjusted to pH 5.8 and solidified with 7.5 $g \cdot L^{-1}$ agar (Sigma-Aldrich Corporation, St. Louis, MO).

Results and Discussion

A total of 3,668 capsules were collected which produced 4,890 seeds and 2,177 viable (non-albino) hybrid seedlings. No fruits or seeds resulted from emasculated/unpollinated flowers from the first self-pollination test. Six taxa produced seeds from the non-emasculated, self-pollination tests, but only two taxa produced viable seedlings. *Syringa pubescens* Tinkerbelle® self-pollinations yielded 36 seeds and 20 viable seedlings. *Syringa meyeri* 'Palabin' self-pollinations yielded one seed and one viable seedling. The following taxa self-pollinations yielded ≤ 3 seeds and no viable seedlings: *S.* ×*hyacinthiflora* 'Old Glory', *S. vulgaris* Blue Skies®, *S. vulgaris* 'Sensation', and *S. vulgaris* 'Tiny Dancer'. Since each self-pollination test was performed on over 200+ flowers per taxon, the chance of self-pollinations was determined to be negligible during controlled crosses.

Viable seedlings across all taxa exhibited a quiescent phase of vegetative growth during their first year. During this period, seedlings produced only a few sets of leaves while they developed an expansive root system (Fig. 2.5A). In the following years, these seedlings exhibited large flushes of vegetative growth. Few phenotypic observations could be made on young seedlings, except for variation in leaf pigments and form. In series *Syringa*, seedlings of parents with lavender or dark purple flowers had a range of abaxial foliar pigment levels. The widest range of color

segregation was observed in *S. vulgaris* ‘Ludwig Spaeth’ x *S. vulgaris* ‘Angel White’ (Fig. 2.2D) where leaf color ranged from green to dark purple. Seedlings with the most purple pigment were from crosses with the purple-leafed cultivar, *S.* ×*hyacinthiflora* ‘Old Glory’ (Fig. 2.2C). All hybrid seedlings exhibited entire margins except for a single seedling from the cross between the double-flowered cultivar *S. vulgaris* ‘President Grevy’ (Fig. 2.1C) and the picotee-flowered cultivar *S. vulgaris* ‘Sensation’ (Fig. 2.1A). This hybrid (H2013-150-001) had leaves with irregular sinuses in its second and third years of growth (Fig. 2.2B). In series *Pubescentes*, one parent, *S. pubescens* subsp. *patula* ‘Miss Kim’, produced only non-viable albino seedlings which failed to survive germination (Fig. 2.5B).

Intraspecific hybridization. Within series *Pubescentes*, a total of 2,180 pollinations resulted in an average of 17.18 ± 11.75 viable seedlings per cross. However, only four crosses yielded viable seedlings out of the 11 attempted. The most prolific cross was between two remontant cultivars, *S. pubescens* Josee™ x *S. pubescens* Bloomerang® Purple, which produced 131 viable seedlings at 0.53 seedlings per pollinated flower (Table 2.3). The reciprocal cross produced 20 viable seedlings at 0.14 seedlings per pollinated flower (Table 2.3). *Syringa pubescens* Josee™ was also an effective seed parent in crosses with *S. pubescens* Tinkerbelle®, yielding 28 viable seedlings at 0.19 seedlings per pollinated flower (Table 2.3). The fewest viable seedlings were produced from the cross *S. pubescens* Tinkerbelle® x *S. pubescens* Bloomerang® with only 10 viable seedlings produced at 0.03 seedlings per pollinated flower (Table 2.3).

Within series *Syringa*, a total of 3,209 pollinations resulted in an average of 24.24 ± 11.28 viable seedlings per cross. Of the 17 crosses attempted, eight yielded viable seedlings. The most prolific cross was *S. vulgaris* 'Ludwig Spaeth' x *S. vulgaris* 'Angel White' producing 186 seedlings at 0.68 seedlings per pollinated flower (Table 2.3). Of the dwarf cultivars, *S. vulgaris* 'Prairie Petite' was the smallest and slowest growing, producing few inflorescences each year. One cross performed with *S. vulgaris* 'Prairie Petite' used 74 pollinations with *S. vulgaris* 'Sensation' and yielded 33 capsules but no seeds (Table 2.3). The dwarf cultivar *S. vulgaris* Tiny Dancer™ was used successfully as both a seed and pollen parent in intraspecific crosses. As a seed parent, *S. vulgaris* Tiny Dancer™ was cross-compatible with *S. vulgaris* 'Sensation' at 0.14 seedlings per pollinated flower, whereas the reciprocal cross yielded 0.21 seedlings per pollinated flower. As a pollen parent, *S. vulgaris* Tiny Dancer™ was also compatible with *S. vulgaris* Blue Skies® at 0.61 seedlings per pollinated flower and *S. vulgaris* 'Lavender Lady' at 0.27 seedlings per pollinated flower (Table 2.3). The double-flowered *S. vulgaris* 'President Grevy' had few successful cross combinations and produced few seedlings. As a seed parent, 807 intraspecific pollinations were performed with four cultivars, and only one seedling was produced from a cross with *S. vulgaris* 'Sensation' (Table 2.3). As a pollen parent, 740 pollinations were performed with *S. vulgaris* 'President Grevy' on three cultivars. Only *S. vulgaris* Blue Skies® proved an effective seed parent, with 12 viable seedlings produced at 0.05 seedlings per pollination (Table 2.3).

Interspecific hybridization. Within series *Pubescentes*, interspecific crosses were performed between cultivars of *S. pubescens* and *S. meyeri* 'Palabin'. A total of

2,649 pollinations resulted in an average of 96.29 ± 37.12 viable seedlings per cross (Table 2.4). Of the seven crosses, only two failed to produce viable seedlings. As a seed parent, *S. pubescens* 'Miss Kim' yielded 149 non-viable, albino seedlings while the reciprocal cross yielded neither viable nor albino seedlings after 522 pollinations (Table 2.4). The most prolific cross, *S. meyeri* 'Palabin' x *S. pubescens* Bloomerang® Purple, yielded 278 seedlings at 1.16 seedlings per pollinated flower (Table 2.4).

Within series *Syringa*, interspecific crosses were performed among cultivars of *S. vulgaris* and *S. ×hyacinthiflora*, as well as wild-type *S. oblata* and *S. oblata* var. *alba*. A total of 3,518 pollinations resulted in an average of 31.71 ± 10.93 viable seedlings per cross (Table 2.4). Of the 24 crosses, 15 resulted in viable seedlings. The most prolific cross was *S. vulgaris* Blue Skies® x *S. ×hyacinthiflora* 'Betsy Ross' producing 1.41 seedlings per pollinated flower (Table 2.4). Although *S. oblata* was successful in a number of interspecific crosses, the white-flowered variety *S. oblata* var. *alba* produced large seedling populations only in crosses with *S. vulgaris* Tiny Dancer™. This cross produced 68 viable seedlings at 0.40 seedlings per pollination while the reciprocal cross produced 47 seedlings at 0.23 seedlings per pollination (Table 2.4).

Not surprisingly, cultivars of *S. ×hyacinthiflora* (hybrids between *S. oblata* and *S. vulgaris*) crossed successfully with elite cultivars of *S. vulgaris* and wild-type *S. oblata*. Crosses with the purple-leafed *S. ×hyacinthiflora* 'Old Glory' (Fig. 2.2C) were of great interest for future breeding within series *Syringa* due to the lack of flower and form diversity in purple leaf cultivars. *Syringa ×hyacinthiflora* 'Old Glory' proved a successful seed parent in crosses with the white-flowered *S. vulgaris*

‘Angel White’, yielding 37 seedlings at 0.19 seedlings per pollinated flower (Table 2.4). Despite 176 pollinations and 76 recovered seeds, *Syringa* ×*hyacinthiflora* ‘Old Glory’ produced no viable seedlings with the double-flowered *S. vulgaris* ‘President Grevy’. When crossed with the dwarf cultivar *S. vulgaris* ‘Tiny Dancer’, *S.* ×*hyacinthiflora* ‘Old Glory’ was an efficient seed parent producing 37 seedlings at 0.30 seedlings per pollination. The reciprocal cross was even more efficient, yielding 160 seedlings at 0.96 seedlings per pollination (Table 2.4).

Within series *Villosae*, only three crosses of the 14 attempted produced seedlings. A total of 2,997 pollinations yielded an average of 9.29 ± 7.19 seedlings per cross combination (Table 2.4). The most prolific cross was *S. julianae* x *S.* ×*prestoniae* ‘Miss Canada’ which produced 100 viable seedlings at 0.48 seedlings per pollinated flower (Table 2.4). *Syringa julianae* had some of the largest flowers in series *Villosae* with fragrance reminiscent of *S. vulgaris*. The only other seed parent to produce viable seedlings in interspecific crosses in series *Villosae* was *S. wolfii*. The cross between *S. wolfii* and yellow-leaved *S. emodii* (Fig. 2.2A) produced 24 seedlings at 0.11 seedlings per pollinated flower (Table 2.4). Only six seedlings resulted from crosses between *S. wolfii* and *S. villosa* yielding 0.03 seedlings per pollinated flower (Table 2.4).

Interseries hybridization. Interseries crosses proved the most challenging because of differences in bloom time. It took three years to complete a range of interseries crosses. A typical lilac cultivar will be in bloom for six weeks in an average season with reliable patterns of bloom across series and species (Fiala, 2008). Our observations agreed with Fiala (2008) with members of series *Syringa* blooming

first in spring, series *Pubescentes* blooming in late spring to early summer, and series *Villosae* blooming in early to mid-summer. During the first year, pollen was collected fresh from early-blooming plants and the sequence of blooming was noted across all accession. Bloom data was used to design reciprocal interseries crosses over the following two years where temperature were altered to speed up or slow down bloom. Earlier bloom times were produced by bringing late flowering taxa into a heated glasshouse in late winter. Later bloom times were produced by monitoring flower progression on early-blooming taxa and placing them in a walk-in cooler to delay anthesis.

From the three series, *Pubescentes, Syringa*, and *Villosae*, there were six possible combinations of interseries crosses (Table 2.5). A total of 41 crosses were performed across these six combinations representing 13,092 total pollinations (Table 2.5). No viable seedlings were recovered although some individual crosses produced capsules and seeds (Table 2.5). Across all the interseries combinations, a total of 975 capsules were recovered which produced 480 seeds. Seed-producing crosses could provide a foundation for future studies on embryo abortion and rescue.

Interseries crosses between *Pubescentes* and *Villosae* included seven crosses that produced both capsules and seeds. These crosses included *S. emodii* and cultivars of *S. pubescens* and *S.* ×*prestoniae* (Table 2.5). Capsules and seeds were produced when taxa in both *Pubescentes* and *Villosae* were used as seed parents. The most prolific cross was *S.* ×*prestoniae* ‘Redwine’ x *S. pubescens* Josee™ which produced 129 seeds from 174 capsules after 602 pollinations (Table 2.5).

Interseries crosses between *Pubescentes* and *Syringa* included five crosses that produced both capsules and seeds. Only one cross produced seeds with series *Syringa* as a seed parent, *S. vulgaris* 'Ludwig Spaeth' x *S. pubescens* Bloomerang®. Only 27 capsules and 18 seeds were recovered from 2,206 pollinations (Table 2.5). Four crosses produced seeds using series *Pubescentes* as a seed parent. The cross *S. oblata* x *S. pubescens* Bloomerang® Purple produce the largest number of capsules of any interseries cross at 238 capsules recovered from 547 pollinations. However, capsules from this cross aborted six weeks after pollination, unlike the majority of interseries capsules which persisted for the majority of the 20 to 30 week fruit development period. The most prolific cross between series *Pubescentes* and *Syringa* was from the cross *S. pubescens* Josee™ x *S. oblata*, yielding 77 seeds from 138 pollinations at 0.56 seed per pollination (Table 2.5).

No interseries crosses between *Syringa* and *Villosae* produced capsules and seeds. Of the 2,016 pollinations, capsules were produced from only three crosses: *S.* ×*hyacinthiflora* 'Old Glory' x *S. villosa*, *S.* ×*hyacinthiflora* 'Old Glory' x *S. wolfii*, and *S. vulgaris* 'Sensation' x *S.* ×*prestoniae* 'Miss Canada' (Table 2.5). The cross that produced the most capsules, *S.* ×*hyacinthiflora* 'Old Glory' x *S. villosa*, yielded 46 capsules from 176 pollinations but no seeds were recovered (Table 2.5).

Embryo rescue. An attempt at extracting open-pollinated lilac seeds from green capsules revealed the difficulty of embryo extraction at this young stage. Oxidation of the young seeds progressed rapidly during excision and phenolics proved damaging when immature seeds were cultured in vitro. Excising seeds while submerged in an anti-oxidant solution containing L-ascorbic acid reduced oxidation

and allowed the culturing of undamaged green seeds. Immature hybrid seeds obtained from interseries crosses in 2013 had low germination percentages in vitro on Monnier's medium (Table 2.6). Over the course of the in vitro germination treatment, the majority of seeds failed to germinate and eventually became necrotic (Fig. 2.4A). Of the 161 seeds cultured in vitro, only three germinated (Fig. 2.4B), all from the cross *S. pubescens* 'Penda' Bloomerang® Purple x *S. vulgaris* 'Ludwig Spaeth' (Table 2.6). The seedlings failed to grow post germination and tissues, including cotyledons, subsequently converted to callus (Fig. 2.4C). This result may be due to lack of proper transfer media post-germination or lack of proper combination of genotype and embryo rescue medium. Surprisingly, Zhou et al. (2003) did not report the lilac genotype used in their protocol. Further research will be necessary to design efficient protocols for in vitro seed germination and embryo rescue of interseries lilac hybrids. Callus obtained from cotyledons in wide lilac hybrids could provide source material for somatic embryogenesis in future studies. Lilac somatic embryogenesis protocols were recently developed for *S. reticulata* var. *mandshurica* (Liu, 2013).

Though interseries crosses and preliminary in vitro germination attempts failed to achieve viable interseries hybrids, the quantity of seeds produced from these wide crosses combined with several seeds that germinated in vitro provides evidence that future work on wide hybridization in lilacs may prove fruitful. Anatomical studies in the past have shown that low germination lilac seeds contain embryos that progress to the walking stick stage before abortion (Chen et al., 2012). This study lists individual cross combinations between series *Pubescentes* and *Villosae*, as well

as *Pubescentes* and *Syringa*, which produced large numbers of seeds (Table 2.5). Current research at North Dakota State University has also found heavy fruit development in interseries crosses between series *Villosae* and the tree lilacs in *Ligustrina* that lasted well into the summer prior to fruit abortion (Nathan Maren, personal communication).

This study represents a comprehensive investigation of lilac cross-compatibility. Intraspecific and interspecific crosses produced hybrid progeny from a diverse set of specific crosses. The resulting seedlings will be used in studying phenotypic segregation of flower traits such as color, double-flowers, picotee petals, and remontancy. Seedlings from crosses with *S. vulgaris* Tiny Dancer™ will be used to study segregation of the dwarf phenotype. Seedlings from crosses *Syringa* ×*hyacinthiflora* 'Old Glory' and *S. emodii* will used to study segregation of the purple and yellow leaf phenotypes, respectively. Selections among hybrid seedlings will identify novel combinations of traits such as purple-leaved dwarfs, double-flowered dwarfs, or double picotee flowers.

Cross-incompatibility, particularly in intraspecific crosses, may be due to differences in genome size and ploidy level. Future work to determine genome size and ploidy differences among taxa, species, and series in *Pubescentes*, *Syringa*, and *Villosae* may provide insight into the cross-compatibility data from the current study. Cross-incompatibility can also be a function of pollination biology. Self-incompatibility systems have been discovered in closely related genera (*Phillyrea*, *Fraxinus*, and *Olea*) and sporophytic cross-incompatibility systems with *S*-allele dominance relationships have been discovered in cultivars of *Olea* (Breton et al.,

2014; Collani et al., 2012; Koubouris et al., 2014; Saumitou-Laprade et al., 2017; Vernet et al., 2016). Future pollination studies investigating rates of gametophytic incompatibility, sporophytic incompatibility, and unreduced gametes may also provide insights into specific cross-compatibility within lilacs. Environmental conditions have also been shown to affect seed development in lilacs (Junttila, 1973), and the current study may provide crosses useful for determining optimum greenhouse temperatures for successful pollinations in lilac.

While many intraspecific and interspecific crosses were successful, no interseries crosses yielded viable progeny. Some interseries crosses did, however, produce multiple capsules and seeds. A preliminary embryo rescue trial yielded low rates of in vitro germination and callus production. These results provide evidence that interseries hybrids among series *Syringa, Pubescentes,* and *Villosae* may be possible. Interseries seed development in hybrids with *Ligustrina* have also shown promise. In future research, seed-producing interseries crosses could be repeated and anatomical studies on embryo development, similar to Chen et al. (2012), may yield important information for targeting future embryo rescue efforts. Open-pollenated seed could be used to identify suitable culture media. Tissue culture and embryo rescue protocols are highly genotype-specific; therefore, cross-specific embryo rescue and embryogenic callus media could be fine-tuned for interseries hybrids that produce viable embryos early in seed development. Recovery of interseries hybrids in lilac will likely prove difficult. However, the current study in combination with other breeding and tissue culture studies provides a foundation for development of novel hybrid lilacs. For a group of ornamental shrubs and trees that have been bred for

nearly 500 years, there are still new horizons for breeders to pursue in modern lilac breeding.

Literature Cited

Bai, C., W.S. Alverson, A. Follansbee, and D.M. Waller. 2012. New reports of nuclear DNA content for 407 vascular plant taxa from the United States. Ann. Bot. (Lond.) 110:1623 – 1629.

Breton, C.M., D. Farinelli, S. Shafiq, J.S. Heslop-Harrison, M. Sedgley, A.J. Bervillé. 2014. The self-incompatibility mating system of the olive (*Olea europaea* L.) functions with dominance between S-alleles. Tree Genet. & Genomes 10: 1055-1067.

Chen, J.-Y., Z.-S. Zhang and D.-Y. Hong. 2009. A taxonomic revision of the *Syringa pubescens* complex (Oleaceae). Ann. Missouri Bot. Garden 96(2): 237-250.

Chen, Y., J.-Y. Chen, Y. Liu and S.-W. Zhao. 2012. Anatomical study on seed abortion of *Syringa villosa* under cultivated conditions. J. Beijing For. Univ. 34(6): 107-114.

Collani, S. G. Galla, A. Ramina, G. Barcaccia, F. Alagna, E.M. Càceres, L. Baldoni, R. Muleo, and G. Perrotta. 2012. Self-incompatibility in Olive: a new hypothesis on the *S*-locus genes controlling pollen-pistil interaction. Acta Hort. 967: 133-140.

Dadapour, M.R., S. Naghiloo, S.H. Peighambardoust, S. Panahirad, M. Aliakbari and A. Movafeghi. 2011. Comparison of floral ontogeny in wild-type and double-flowered phenotypes of *Syringa vulgaris* L. (Oleaceae). Sci. Hort. 127: 535-541.

Fiala, J.L. Lilacs. The Genus *Syringa*. 1988. Timber Press, Inc., Portland, OR.

Fiala, J.L. and F. Vrugtman. 2008. Lilac: A Gardener's Encyclopedia. 2nd ed. Timber Press, Inc., Portland, OR.

Greilhuber, J., E.M. Temsch, and J.C.M. Loureiro. 2007. Nuclear DNA content measurement, p. 67 – 101. In: Doležel, J., J. Greilhuber, and J. Suda (eds.). Flow cytometry with plant cells: Analysis of genes, chromosomes and genomes. WILEY-VCH Verlag GmbH & Co., KGaA, Weinheim, Germany.

Hirtz, F. 1993. Victor Lemoine, un grand nom de l'horticulture mondiale. Thesis, Diplôme d'Etat de Docteur en Pharmacie, Université de Nancy.

Junttila, O. 1973. Seeds and embryo germination in *Syringa vulgaris* and *S. reflexa* as affected by temperature during seed development. Physiol. Plant. 29: 264-268.

Kim, K.-J. and R.K. Jansen. 1998. A chloroplast DNA phylogeny of lilacs (*Syringa*, Oleaceae): plastome groups show a strong correlation with crossing groups. Amer. J. Bot. 85(9): 1338-1351.

Kochieva, E.Z., N.N. Ryzhova, O.I. Molkanova, A.M. Kudryavtsev, V.P. Upelniek and I.B. Okuneva. 2004. The Genus *Syringa*: molecular markers of species and cultivars. Russian J. Genet. 40(1): 30-32.

Koubouris, G.C., C.M. Breton, I.T. Metzidakis, and M.D. Vasilakakis. 2014. Self-incompatibility and pollination relationships for four Greek olive cultivars. Scientia Hort. 176: 91-96.

Lemoine, V. 1878. *Syringa hyacinthiflora plena*. Catalogue 78, p.6. Nancy, France.

Li, J., B. Goldman-Huertas, J. DeYoug and J. Alexander, III. 2012. Phylogenetics and diversification of *Syringa* inferred from nuclear and plastid DNA sequences. Castanea 77(1): 82-88.

Monnier, M. 1990. Culture of Zygotic Embryos of Higher Plants. Methods Mol. Biol. 6: 129-139.

Pringle, J.S. 1981. A review of attempted and reported interseries and intergeneric hybridization in *Syringa* (Oleaceae). Baileya 20: 49-91.

Rehder, A. 1945. Notes on some cultivated trees and shrubs. J. Arnold Arboretum 26: 67-78.

Saumitou-Laprade, P., P. Vernet, X. Vekemans, S. Billiard, S. Gallina, L. Essalouh, A. Mhaïs, A. Moukhli, A. El Bakkali, G. Barcaccia, F. Alagna, R. Mariotti, N.G.M. Cultrera, S. Pandolfi, M. Rossi, B. Khadari, and L. Baldoni. 2017. Elucidation of the genetic architecture of self-incompatibility in olive: evolutionary consequences and perspectives for orchard management. Evolutionary Applications (Accepted): DOI: 10.1111/eva.12457.

U.S. Department of Agriculture (USDA). 2016. Census of horticultural specialties for 2014. 15 July 2016. https://www.agcensus.usda.gov/Publications/2012/Online_Resources/Census_of_Horticulture_Specialties/

Vernet, P., P. Lepercq, S. Billiard, A. Bourceaux, J. Lepart, B. Dommée and Pierre Saumitou-Laprade. 2016. Evidence for the long-term maintenance of a rare self-incompatibility system in Oleaceae. New Phytol. 210: 1408-1417.

Zhou, L., L.-M. Dai and B.-L. Su. 2003. Embryo culture and rapid propagation of *Syringa*. J. For. Res. 14(3): 213-216.

Tables

Table 2.1. Interspecific hybrids in lilac and their parent species (Fiala and Vrugtman, 2008).

Interspecific Hybrid	Parent 1	Series	Parent 2	Series
Syringa ×*chinensis*	*S. protolaciniata*	*Syringa*	*S. vulgaris*	*Syringa*
Syringa ×*diversifolia*[z]	*S. pinnatifolia*	*Pinnatifoliae*	*S. oblata* subsp. *oblata*	*Syringa*
Syringa ×*henryi*	*S. josikaea*	*Villosae*	*S. villosa*	*Villosae*
Syringa ×*hyacinthiflora*	*S. oblata*	*Syringa*	*S. vulgaris*	*Syringa*
Syringa ×*josiflexa*	*S. josikaea*	*Villosae*	*S. reflexa*	*Villosae*
Syringa ×*laciniata*	unknown	unknown	unknown	unknown
Syringa ×*nanceiana*	*Syringa* ×*henryi*	*Villosae*	*Syringa sweginzowii*	*Villosae*
Syringa ×*persica*	unknown	unknown	unknown	unknown
Syringa ×*prestoniae*	*S. villosa*	*Villosae*	*S. komarowii*	*Villosae*
Syringa ×*swegiflexa*	*S. komarowii*	*Villosae*	*S. sweginzowii*	*Villosae*

[z]Interseries hybrid

Table 2.2. Source material for lilac breeding at Oregon State University.

Series[z]	Taxon[y]	Cultivar / Trademark Name[x]	Accession[w]	Source[v]
Syringa	*S. oblata*		09-0058	Arborétum Mlyňany
	S. oblata var. *alba*		09-0059	Arborétum Mlyňany
	S. vulgaris	'Agincourt Beauty'	13-0036	Briggs Nursery
		'Agincourt Beauty'	14-0124	Dennis' 7 Dees
		'Angel White'	10-0043	Blue Heron Farm
		'Angel White'	13-0075	Monrovia
		'Monore' Blue Skies®	13-0076	Monrovia
		'Lavendar Lady'	13-0078	Monrovia
		'Ludwig Spaeth'	10-0042	Blue Heron Farm
		'Ludwig Spaeth'	13-0079	Monrovia
		'Prairie Petite'	13-0035	Briggs Nursery
		'President Grevy'	10-0040	Blue Heron Farm
		'President Grevy'	14-0125	Portland Nursery
		'President Lincoln'	13-0080	Monrovia
		'Sensation'	13-0081	Monrovia
		'Elsdancer' Tiny Dancer™	13-0001	Heritage Seedlings
	S. ×*hyacinthiflora*	'Betsy Ross'	13-0034	Briggs Nursery
		'Maiden's Blush'	14-0123	Dennis' 7 Dees
		'Old Glory'	13-0085	Monrovia
		'Pocahontas'	13-0084	Monrovia
Pubescentes	*S. meyeri*	'Palabin'	10-0209	Bailey Nurseries
	S. pubescens	'Penda' Bloomerang® Purple	12-0026	Garland Nursery
		'Penda' Bloomerang® Purple	13-0070	Monrovia
		'Penda' Bloomerang® Purple	14-0189	Select Plus
		'SMSJBP7' Bloomerang® Dark Purple	13-0071	Monrovia
		'MORjos 060F' Josee™	10-0039	Blue Heron Farm
		'Bailbelle' Tinkerbelle®	12-0027	Bailey Nurseries
	S. pubescens subsp. *patula*	'Miss Kim'	13-0073	Monrovia

Table 2.2 (continued). Source material for lilac breeding at Oregon State University.

Series[z]	Taxon[y]	Cultivar / Trademark Name[x]	Accession[w]	Source[v]
Villosae	*S. emodii*		09-0038	Hohenheim Gardens
	S. josikaea		09-0039	Hohenheim Gardens
	S. julianae		09-0057	Arborétum Mlyňany
	S. sweginzowii		11-0021	NBG Dublin
	S. tigerstedtii		09-0040	Hohenheim Gardens
	S. villosa		09-0061	Arborétum Mlyňany
			10-0020	Rogów Arboretum
	S. wolfii		09-0062	Mlynany Arboretum
			10-0021	Rogów Arboretum
	S. ×prestoniae	'Miss Canada'	13-0037	Briggs Nursery
		'Miss Canada'	13-0087	Monrovia
		'Redwine'	13-0088	Monrovia
	S. yunnanensis		09-0063	Arborétum Mlyňany

[z]Series designation based on phylogeny by Li et al. (2012).

[y]Individual taxon in *Syringa* (L.) based on current phylogeny (Li et al.. 2012) and revisions (Chen et al., 2009).

[x]Cultivar and trademark name.

[w]Accession number in research collection at Oregon State University, Corvallis, OR. Duplicate samples were clones and phenotypically identical.

[v]Container plants, seeds, and leaf samples collected from the following sources: Arborétum Mlyňany, Slepcany, Slovakia; Bailey Nurseries, Yamhill, OR; Blue Heron Farm, Corvallis, OR; Briggs Nursery, Elma, WA; Carlton Plants, Dayton, OR; Dennis' 7 Dees Landscaping & Garden Centers, Portland, OR; Garland Nursery, Corvallis, OR; Heritage Seedlings & Liners, Salem, OR; Hohenheim Gardens, Stuttgart, Germany; Mason Hollow Nursery, Mason, NH; Monrovia, Dayton, OR; National Botanic Gardens (Dublin), Glasnevin, Ireland; Portland Nursery, Portland, OR; Rogów Arboretum (Arboretum SGGW w Rowie), Rogów, Poland; Select Plus International Lilac Nursery, Quebec, Canada.

Table 2.3. Intraspecific cross-compatibility within series *Pubescentes* and *Syringa* in lilac.

Series[z]	Female Parent	Male Parent	Pollinations[y]	Capsules[x]	Seed[w]	Germinated[v]
Pubescentes						
	S. pubescens Bloomerang® Purple	*S. pubescens* Josee™	141	28	41	20
		S. pubescens 'Miss Kim'	175	1	1	0
		S. pubescens Tinkerbelle®	133	0	0	0
	S. pubescens Josee™	*S. pubescens* Bloomerang® Purple	246	67	158	131
		S. pubescens 'Miss Kim'	137	4	5	0
		S. pubescens Tinkerbelle®	145	31	42	28
	S. pubescens 'Miss Kim'	*S. pubescens* Bloomerang® Purple	380	56	58	11[u]
		S. pubescens Josee™	210	0	0	0
	S. pubescens Tinkerbelle®	*S. pubescens* Bloomerang® Purple	290	20	15	10
		S. pubescens Josee™	199	0	0	0
		S. pubescens 'Miss Kim'	124	0	0	0
Syringa						
	S. vulgaris Tiny Dancer™	*S. vulgaris* 'Angel White'	270	3	0	0
		S. vulgaris 'President Lincoln'	61	6	2	0
		S. vulgaris 'Sensation'	125	18	28	18
	S. vulgaris 'Angel White'	*S. vulgaris* 'Ludwig Spaeth'	353	153	204	55
	S. vulgaris Blue Skies®	S. *vulgaris* Tiny Dancer™	100	81	160	61
		S. vulgaris 'President Grevy'	238	68	72	12
	S. vulgaris 'Lavender Lady'	*S. vulgaris* Tiny Dancer™	176	75	93	47
	S. vulgaris 'Ludwig Spaeth'	*S. vulgaris* 'Angel White'	273	182	422	186
	S. vulgaris 'Prairie Petite'	*S. vulgaris* 'Sensation'	74	33	0	0
	S. vulgaris 'President Grevy'	S. *vulgaris* Tiny Dancer™	304	3	2	0
		S. vulgaris 'Angel White'	182	4	3	0
		S. vulgaris 'President Lincoln'	81	0	0	0
		S. vulgaris 'Sensation'	240	100	107	1
	S. vulgaris 'President Lincoln'	*S. vulgaris* 'Angel White'	135	27	23	12
		S. vulgaris 'President Grevy'	126	0	0	0
	S. vulgaris 'Sensation'	S. *vulgaris* Tiny Dancer™	95	12	14	20
		S. vulgaris 'President Grevy'	376	16	13	0

[z]Intraspecific crosses within two series of lilac: *Pubescentes* and *Syringa* (Li et al., 2012).
[y]Number of emasculated flowers pollinated.
[x]Number of capsules formed from controlled crosses.
[w]Number of seed produced from controlled crosses.
[v]Number of seed germinated.
[u]All nonviable albino seedlings.

Table 2.4. Interspecific cross-compatibility within series *Pubescentes*, *Syringa*, and *Villosae* in lilac.

Series[z]	Female Parent	Male Parent	Pollinations[y]	Capsules[x]	Seed[w]	Germinated[v]
Pubescentes						
	S. meyeri 'Palabin'	*S. pubescens* Bloomerang® Purple	239	144	398	278
		S. pubescens Josee™	706	47	47	19
		S. pubescens 'Miss Kim'	522	24	39	0
		S. pubescens Tinkerbelle®	206	83	155	134
	S. pubescens Josee™	*S. meyeri* 'Palabin'	122	38	82	63
	S. pubescens 'Miss Kim'	*S. meyeri* 'Palabin'	601	417	900	149[u]
	S. pubescens Tinkerbelle®	*S. meyeri* 'Palabin'	253	64	58	31
Syringa						
	S. oblata	*S. vulgaris* Tiny Dancer™	92	13	14	10
		S. vulgaris 'Lavender Lady'	162	0	0	0
	S. *oblata* var. *alba*	*S. vulgaris* Tiny Dancer™	208	53	75	47
		S. vulgaris 'President Lincoln'	226	0	0	0
	S. vulgaris Tiny Dancer™	*S. oblata*	175	17	10	8
		S. *oblata* var. *alba*	170	59	75	68
		S. ×*hyacinthiflora* 'Old Glory'	166	96	172	160
		S. ×*hyacinthiflora* 'Pocahontas'	32	6	0	0
	S. vulgaris 'Agincourt Beauty'	*S.* ×*hyacinthiflora* 'Old Glory'	116	56	81	6
	S. vulgaris Blue Skies®	S. *oblata* var. *alba*	103	5	5	4
		S. ×*hyacinthiflora* 'Betsy Ross'	135	103	214	191
	S. vulgaris 'Lavender Lady'	*S. oblata*	131	48	67	49
		S. *oblata* var. *alba*	155	6	2	0
	S. vulgaris 'Prairie Petite'	*S.* ×*hyacinthiflora* 'Old Glory'	20	0	0	0
	S. vulgaris 'President Grevy'	*S.* ×*hyacinthiflora* 'Old Glory'	176	59	76	0
	S. vulgaris 'Sensation'	*S.* ×*hyacinthiflora* 'Old Glory'	173	73	0	0
	S. ×*hyacinthiflora* 'Betsy Ross'	*S. oblata*	79	2	2	1
		S. *oblata* var. *alba*	112	8	8	5
	S. ×*hyacinthiflora* 'Maiden's Blush'	*S. oblata*	164	11	15	13
	S. ×*hyacinthiflora* 'Old Glory'	*S. oblata*	234	70	154	125
		S. *oblata* var. *alba*	290	0	0	0
		S. *vulgaris* Tiny Dancer™	122	29	42	37
		S. vulgaris 'Angel White'	195	57	95	37
		S. vulgaris 'Sensation'	82	0	0	0

Table 2.4 (continued). Interspecific cross-compatibility within series *Pubescentes*, *Syringa*, and *Villosae* in lilac.

Series[z]	Female Parent	Male Parent	Pollinations[y]	Capsules[x]	Seed[w]	Germinated[v]
Villosae						
	S. emodii	*S.* ×*prestoniae* 'Miss Canada'	240	0	0	0
		S. villosa	213	0	0	0
		S. wolfii	309	0	0	0
		S. yunnanensis	243	0	0	0
	S. julianae	*S.* ×*prestoniae* 'Miss Canada'	255	69	122	100
	S. villosa	*S.* ×*prestoniae* 'Miss Canada'	181	0	0	0
		S. emodii	169	0	0	0
		S. wolfii	179	0	0	0
	S. wolfii	*S.* ×*prestoniae* 'Miss Canada'	175	0	0	0
		S. emodii	215	31	25	24
		S. villosa	178	11	14	6
	S. yunnanensis	*S. julianae*	177	0	0	0
		S. ×*prestoniae* 'Miss Canada'	209	0	0	0
	S. ×*prestoniae* 'Miss Canada'	*S. wolfii*	254	0	0	0

[z]Interspecific crosses within three series of lilac: *Pubescentes*, *Syringa*, and *Villosae* (Li et al., 2012).
[y]Number of emasculated flowers pollinated.
[x]Number of capsules formed from controlled crosses.
[w]Number of seed produced from controlled crosses.
[v]Number of seed germinated.
[u]Nonviable albino seedlings produced.

Table 2.5. Interseries cross-compatibility among series *Pubescentes*, *Syringa*, and *Villosae* in lilac.

Interseries Cross[z]	Female Parent	Male Parent	Pollinations[y]	Capsules[x]	Seed[w]	Germinated[v]
Pubescentes x *Villosae*						
	S. pubescens Bloomerang® Purple	*S.* ×*prestoniae* 'Miss Canada'	482	17	8	0
	S. pubescens Josee™	*S.* ×*prestoniae* 'Miss Canada'	500	65	49	0
		S. ×*prestoniae* 'Redwine'	150	1	1	0
Villosae x *Pubescentes*						
	S. emodii	*S. pubescens* Bloomerang® Dark Purple	82	21	14	0
		S. pubescens Bloomerang® Purple	97	0	0	0
	S. josikaea	*S. meyeri* 'Palabin'	58	0	0	0
		S. pubescens Josee™	149	6	0	0
		S. pubescens Tinkerbelle®	135	10	0	0
	S. julianae	*S. pubescens* Bloomerang® Dark Purple	64	16	0	0
	S. sweginzowii	*S. pubescens* Bloomerang® Dark Purple	237	22	0	0
	S. tigerstedii	*S. pubescens* Bloomerang® Dark Purple	130	12	0	0
	S. villosa	*S. pubescens* Bloomerang® Dark Purple	219	0	0	0
	S. wolfii	*S. pubescens* Bloomerang® Purple	176	0	0	0
	S. yunnanensis	*S. pubescens* Bloomerang® Dark Purple	163	0	0	0
	S. ×*prestoniae* 'Miss Canada'	*S. pubescens* Bloomerang® Purple	425	80	73	0
	S. ×*prestoniae* 'Redwine'	*S. pubescens* Bloomerang® Purple	617	56	44	0
		S. pubescens Josee™	602	174	129	0
Pubescentes x *Syringa*						
	S. meyeri 'Palabin'	*S. oblata*	179	10	6	0
		S. vulgaris 'Angel White'	91	0	0	0
		S. vulgaris 'Sensation'	197	55	39	0
	S. pubescens Bloomerang® Purple	*S. vulgaris* 'Ludwig Spaeth'	2098	31	21	3[u]
	S. pubescens Josee™	*S. oblata*	138	60	77	0
		S. oblata var. *alba*	329	1	1	0
	S. pubescens 'Miss Kim'	*S. oblata*	223	0	0	0
		S. vulgaris 'President Grevy'	408	0	0	0
	S. pubescens Tinkerbelle®	*S. oblata*	271	0	0	0
Syringa x *Pubescentes*						
	S. oblata	*S. pubescens* Bloomerang® Purple	547	238	0	0
	S. vulgaris 'Ludwig Spaeth'	*S. pubescens* Bloomerang® Purple	2206	27	18	0
	S. vulgaris 'President Grevy'	*S. pubescens* Bloomerang® Purple	68	27	0	0
	S. ×*hyacinthiflora* 'Old Glory'	*S. pubescens* Bloomerang® Purple	35	0	0	0

Table 2.5 (continued). Interseries cross-compatibility among series *Pubescentes*, *Syringa*, and *Villosae* in lilac.

Interseries Cross[z]	Female Parent	Male Parent	Pollinations[y]	Capsules[x]	Seed[w]	Germinated[v]
Syringa x *Villosae*						
	S. ×*hyacinthiflora* 'Old Glory'	*S. villosa*	176	46	0	0
		S. wolfii	248	1	0	0
		S. yunnanensis	153	0	0	0
		S. ×*prestoniae* 'Miss Canada'	305	0	0	0
	S. vulgaris 'President Grevy'	*S.* ×*prestoniae* 'Miss Canada'	22	0	0	0
	S. vulgaris 'Sensation'	*S. julianae*	172	0	0	0
		S. villosa	142	0	0	0
		S. wolfii	149	0	0	0
		S. ×*prestoniae* 'Miss Canada'	232	5	0	0
Villosae x *Syringa*						
	S. ×*prestoniae* 'Miss Canada'	*S. vulgaris* 'Sensation'	295	0	0	0
	S. wolfii	*S. vulgaris* Blue Skies®	122	0	0	0

[z]Interseries crosses representing six reciprocal combinations of three series of lilac: *Pubescentes*, *Syringa*, and *Villosae* (Li et al., 2012).
[y]Number of emasculated flowers pollinated.
[x]Number of capsules formed from controlled crosses.
[w]Number of seed produced from controlled crosses.
[v]Number of seed germinated.
[u]Attempted vitro germination of seed on Monnier's Medium according to Zhou et al. (2003); seedlings did not survive germination.

Table 2.6. Attempted pollinations, recovered seed, and in vitro germination of interseries lilac hybrids in 2013. All seed collected from green capsules and cultured on cultured on Monnier's medium as described by Zhou et al. (2003).

Female Parent	Male Parent	Pollinated flowers	Seed	Germinated
S. vulgaris 'Ludwig Spaeth'	*S. pubescens* Bloomerang® Purple	2206	18	0
S. oblata	*S. pubescens* Bloomerang® Purple	547	0[z]	0
S. meyeri 'Palabin'	*S. oblata*	179	6	0
	S. vulgaris 'Angel White'	91	0	0
	S. vulgaris 'Sensation'	197	39	0
S. pubescens 'Miss Kim'	*S. oblata*	223	0	0
	S. vulgaris 'President Grevy'	408	0	0
S. pubescens Josee™	*S. oblata*	138	77	0
S. pubescens Bloomerang® Purple	*S. vulgaris* 'Ludwig Spaeth'	2098	21	3[y]

[z]Early abortion of 238 fruit occurred six weeks post-pollination

[y]Radicle, hypocotyl, and cotyledons emerged; seedlings failed to grow post-germination and tissues subsequently converted to callus.

Figures

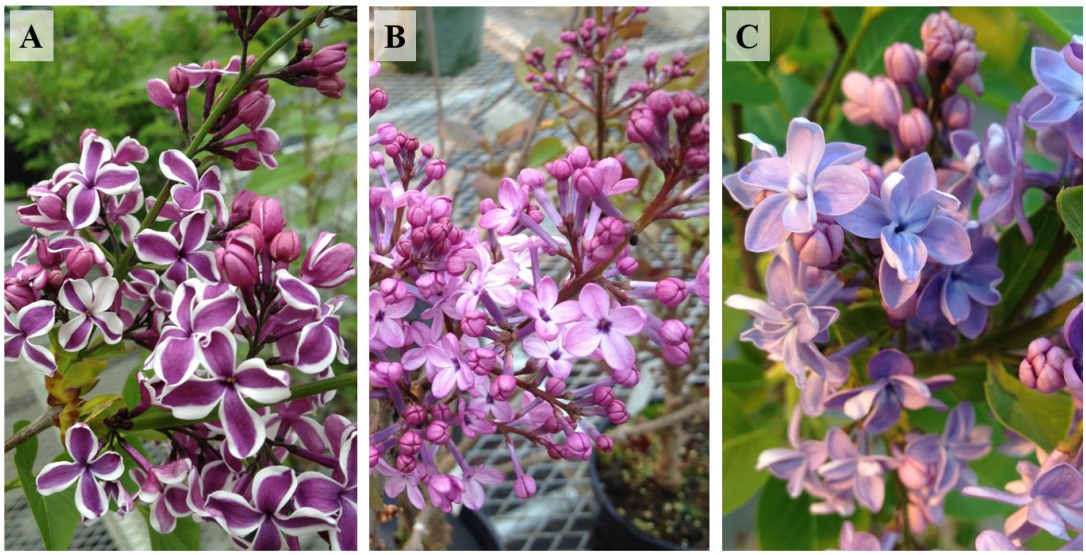

Fig. 2.1. Flower form phenotypes in lilac breeding population at Oregon State University. (A) Picotee flower of *Syringa vulgaris* ‘Sensation’. (B) Single flower form of *Syringa* ×*hyacinthiflora* ‘Old Glory’. (C) Hose-in-hose flower of *Syringa vulgaris* ‘Old Glory’.

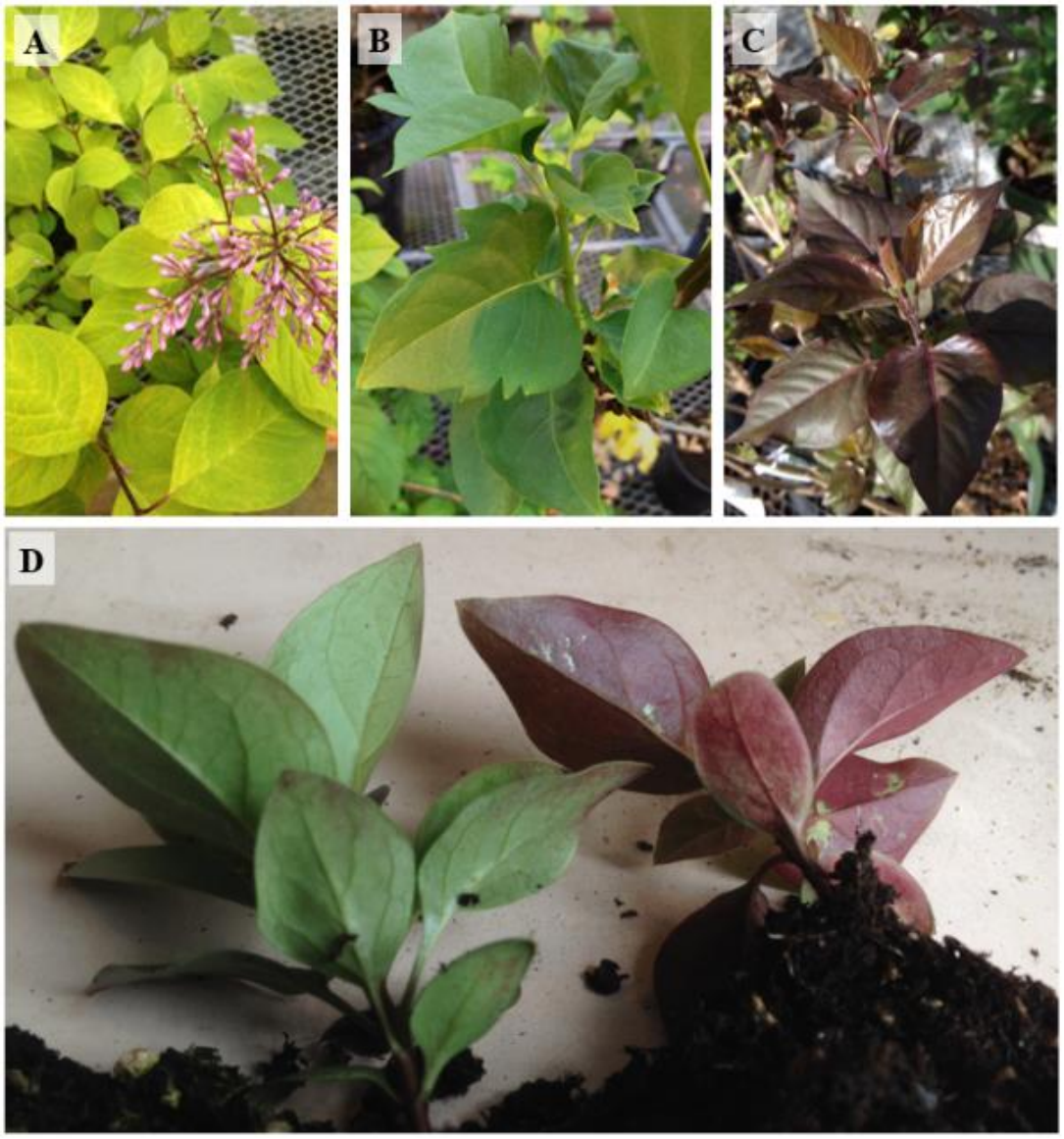

Fig. 2.2. Spring leaf color phenotypes in lilac breeding population at Oregon State University. (A) Yellow-green leaves of *Syringa emodii* (B) Hybrid seedling (H2013-150-001) from the cross *Syringa vulgaris* 'President Grevy' x *Syringa vulgaris* 'Sensation' (C) *Syringa* ×*hyacinthiflora* 'Old Glory' (D) Segregating hybrid seedlings from the cross *Syringa vulgaris* 'Ludwig Spaeth' x Syringa vulgaris 'Angel White'.

Fig. 2.3. Breeding cycle for lilacs over one year at Oregon State University. (A) Pollen was collected fresh in spring and stored in a 4 °C refrigerator over desiccant. (B) Open flowers were removed and the remaining flowers were emasculated prior to two to three pollinations over consecutive days. (C) In summer, fruits were counted, collected, and allowed to dry before extracting seed in fall. (D) Seeds were cold stratified for ten weeks at 4 °C and sown in 1.3 L containers filled with a peat-based substrate during winter with ≤30 seeds per pot. (E) Hybrid seedlings were grown in tree tubes trays (0.22 L per cell) before transplanting to 2.5-L containers in the spring.

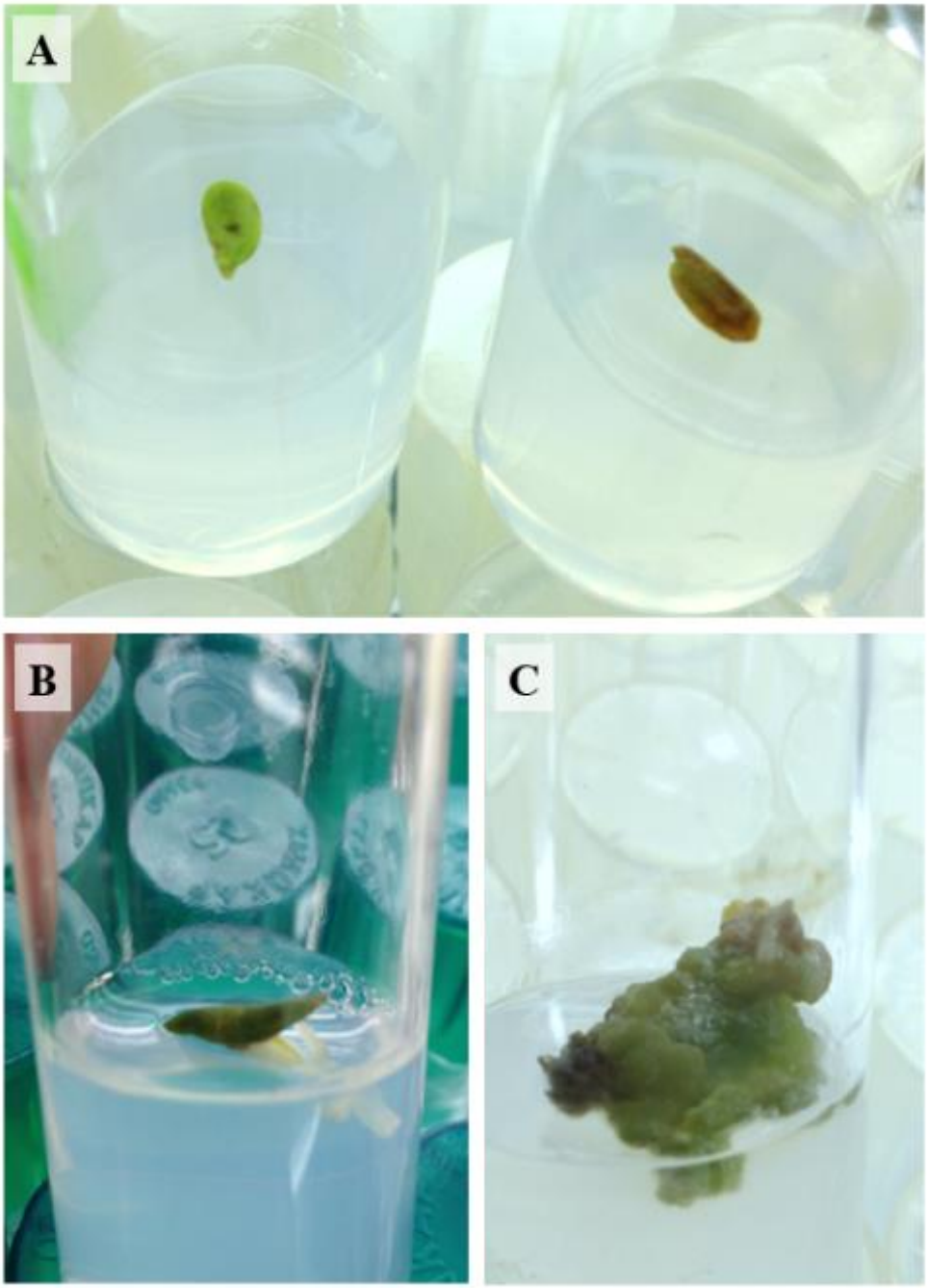

Fig. 2.4. (A) Variation in seed survival and germination in vitro (left green, right brown). (B) Germination in vitro of interseries hybrid seedling from cross S. *pubescens* subsp. *pubescens* 'Penda' Bloomerang® Purple x *Syringa vulgaris* 'Ludwig Spaeth' (C) Post-germination callus development on interseries hybrid seedling of the same cross.

Fig. 2.5. Post-stratification seed germination in hybrid population at Oregon State University. (A) Root growth of quiescent first-year seedling from the cross *Syringa vulgaris* ‘Agincourt Beauty’ x *Syringa* ×*hyacinthiflora* ‘Old Glory’ (B) Non-viable albino seedlings from crosses of *Syringa pubescens* subsp. *patula* ‘Miss Kim’ x *Syringa meyeri* ‘Palabin’.

CHAPTER 3: PLOIDY AND GENOME SIZE IN LILAC SPECIES, CULTIVARS, AND INTERPLOID HYBRIDS

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CHAPTER 3: PLOIDY AND GENOME SIZE IN LILAC SPECIES, CULTIVARS, AND INTERPLOID HYBRIDS.

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Additional index words: flow cytometry, cytology, triploid, aneuploidy, unreduced gametes

Abstract. Genome size variation can be used to investigate biodiversity, genome evolution, and taxonomic relationships among related taxa. Plant breeders use genome size variation to identify parents useful for breeding sterile or improved ornamentals. Lilacs (*Syringa*) are deciduous trees and shrubs valued for their fragrant spring and summer flowers. The genus is divided into six series: *Syringa* (=*Vulgares*), *Pinnatifoliae*, *Ligustrae*, *Ligustrina*, *Pubescentes* and *Villosae*. Reports conflict on genome evolution, base chromosome number, and polyploidy in lilac. The purpose of this study was to investigate genome size and ploidy variation across a diverse collection. Flow cytometry was used to estimate holoploid (2C) genome sizes (relative to an internal standard) in series, species, cultivars, and seedlings from parents with three ploidy combinations: 2*x* x 2*x*, 2*x* x 3*x*, and 3*x* x 2*x*. Monoploid (1C*x*) genome sizes were calculated by dividing 2C genome size by ploidy, which was confirmed in a subset of taxa using root tip microscopy. Pollen diameter was measured to investigate the frequency of unreduced gametes in diploid and triploid *S. vulgaris* cultivars. Three triploids of *S. vulgaris* were observed: 'Aucubaefolia',

‘Agincourt Beauty’, and ‘President Grévy’. Across taxa, significant variations in 1C*x* genome size were discovered. The smallest and largest values were found in the interspecific hybrids *S.* ×*laciniata* (1.32 ± 0.04 pg) and *S.* ×*hyacinthiflora* ‘Old Glory’ (1.78 ± 0.05), both of which are in series *Syringa*. Series *Syringa* (1.68 ± 0.02 pg) had a significantly larger 1C*x* genome size than the other series. No significant differences were found within series *Pubescentes* (1.47 ± 0.01 pg), *Villosae* (1.55 ± 0.02 pg), *Ligustrina* (1.49 ± 0.05 pg), and *Pinnatifoliae* (1.52 ± 0.02 pg). For *S. vulgaris* crosses, no significant variation in 2C genome size was discovered in 2*x* x 2*x* crosses. Interploid crosses between ‘Blue Skies’ (2*x*) and ‘President Grévy’ (3*x*) produced an aneuploid population with variable 2C genome sizes ranging from 3.41 ± 0.03 pg to 4.35 ± 0.03 pg. Only one viable seedling was recovered from a cross between ‘President Grévy’ (3*x*) and ‘Sensation’ (2*x*). This seedling had a larger 2C genome size (5.65 ± 0.02 pg) than either parent, and the largest 2C genome size currently reported in lilac. Pollen diameter measurements revealed that ‘Sensation’ produced 8.5% unreduced pollen, which we inferred was responsible for the increased genome size. No unreduced pollen was discovered in the other diploids examined. Increased ploidy may provide a mechanism for recovering progeny from incompatible taxa in lilac breeding.

Introduction

Genome size variation can be used to investigate biodiversity, taxonomic relationships, and genome evolution among related taxa (Greilhuber, 1998; Rounsaville and Ranney, 2010; Shearer and Ranney, 2013; Zonneveld and Duncan,

2010; Zonneveld et al., 2005). Studies on genome evolution focus on large, structural changes in sequence or fluctuations in genome size resulting from natural phenomena including polyploidy, chromosome fission/fusion, and interploid hybridization (Soltis and Soltis, 2012). Genome size variation can also be used by plant breeders to identify parents for wide hybrids among parent taxa. Interspecific hybrids have been shown to have genome sizes intermediate between their parents in other woody ornamentals such as dogwood (*Cornus*; Shearer and Ranney, 2013) and magnolia (*Magnolia*; Parris et al., 2010). When combining genome sizes with their corresponding chromosome counts, genome size data can be used to discover ploidy variation among related taxa (Contreras et al., 2013; Lattier, 2016; Parris et al., 2010; Shearer and Ranney, 2013). Polyploidy, or whole genome duplication, is a driving force in evolution and occurs naturally through somatic mutations in meristematic cells and through unreduced gametes (Harland and deWet, 1975; Ranney, 2006). There are two broad categories of polyploidy; autopolyploidy is the duplication of a single genome while allopolyploidy is the combination of two or more different genomes and an associated duplication event (Chen and Ni, 2006).

Identification and induction of polyploidy can be valuable tools for plant breeding. Irregular meiosis in gametes can result in sterility, while "gigas" effects of somatic cells can result in thicker, glossier cuticles, enlarged flowers, or enlarged fruit (Ranney, 2006). In addition, polyploids have been used to overcome interploid hybridization barriers (Ranney, 2006) and to restore fertility in wide hybrids of ornamentals such as *Rhododendron* 'Fragrant Affinity' and ×*Chitalpa tashkentensis* (Contreras et al., 2007; Olsen et al., 2006).

Lilacs (*Syringa*) are a genus of deciduous, woody trees and shrubs grown primarily for their heavy spring and summer blooms of fragrant flowers. *Syringa* is comprised of 21 to 28 species that are part of the monophyletic subfamily Oleoideae in family Oleaceae and are closely allied with *Ligustrum* (Li et al., 2002; Wallander and Albert, 2000). Recent taxonomic studies divide the genus into six series: *Syringa, Pinnatifoliae, Ligustrae, Ligustrina, Pubescentes,* and *Villosae* (Li et al., 2012). Most species are native to eastern Asia while two species, *S. vulgaris* and *S. josikaea,* are native to southeastern Europe (Kim and Jansen, 1998). Most cultivar development over centuries of breeding has focused on improvements of common lilac (*S. vulgaris*) within series *Syringa*.

Lilacs have proven to be important ornamental crops, yet little is known about how nuclear genome varies among series, species, hybrids, and cultivars. A survey of genome size (C-value) and ploidy level within *Syringa* would contribute to the call for a global census of angiosperm C-values (Galbraith et al., 2011). Though genome sequencing is a powerful tool for studying gene function, C-values calculated from sequencing data tend to underestimate true genome size (relative to flow cytometry) due to misassembly and the inability to sequence through repetitive regions of the genome (Bennett and Leitch, 2011). Flow cytometry measurements of genome size have proven useful for identification of species, hybrids, polyploids, and polyploid series (Galbraith et al., 2011).

In genera such as lilac with a long history of breeding and cultivation, variation in genome size and chromosome number can occur from interspecific hybridization, unreduced gametes, and induction of autopolyploids. Interspecific

hybridization has been a valuable tool for producing many new cultivars of lilac (Table 3.1). Two reports on genome size estimates in lilac focused on two European species, *S. vulgaris* and *S. josikaea*. Siljak-Yakovlev et al. (2010) reported *S. vulgaris* to have a 2C genome size of 2.4 pg based on propidium iodide flow cytometry. Olszewska and Osiecka (1984) reported *S. josikaea* to have a 2C genome size of 2.6 pg based on Feulgen cytophotometry. Despite the paucity of genome size estimates in lilac, much effort has been dedicated to studying chromosome number variation in lilac and the Oleaceae.

Phylogenetic analysis has determined the ancestral state of the Oleaceae to be diploid (Taylor, 1945). Cyto-taxonomy divides the Oleaceae into two groups according to basic chromosome number with the first group consisting of *Mendora* (x = 11), *Jasminum* (x = 13), *Fontanesia* (x = 13), *Forsythia* (x = 14), and *Abeliophyllum* (x = 14). The second group (originally designated as subfamily Oleoideae) consists of genera with x = 23, including *Olea*, *Syringa*, *Ligustrum*, *Fraxinus*, *Osmanthus*, *Forestiera*, *Phillyrea*, *Osmarea*, and *Chionanthus* (Taylor, 1945). Lilacs are primarily diploids with basic chromosome numbers reported at x = 22, 23, or 24 (Darlington and Wylie, 1956). Sax (1930) reported the "fundamental" chromosome number in lilac to be x = 12 and hypothesized that ancestral polyploidization of an x = 11 or x = 12 cytotype was responsible for the variation in chromosome numbers, with the x = 23 cytotype resulting from the loss of a pair of chromosomes. In contrast, Taylor (1945) reported that most wild-type lilac specimens are x = 23 cytotypes, not x = 22 or x = 24. The prevalence and stability of the x = 23 cytotype throughout the Oleaceae, illustrated by Taylor (1945) and Stebbins (1940), indicates that the x = 23

cytotype likely predates other cytotypes in *Syringa*, and originated as the result of allopolyploidy between ancestral Oleaceae taxa of two cytotypes, $x = 11$ and $x = 12$. Therefore, the variation in chromosome number observed in common lilac is likely the result of aneuploidy over centuries of plant collection and wide hybridization.

Aside from theories of ancestral allopolyploidy, no reports exist to confirm polyploidy in wild or cultivated lilac populations. In addition, no reports of natural polyploidy exist for the closely related genera *Ligustrum*. However, natural polyploidy has been discovered in other related genera in Oleaceae. Taylor (1945) reported tetraploids in *Mendora*, tetraploids and triploids in *Jasminum*, tetraploids and hexaploids in *Fraxinus*, and hexaploids in *Osmanthus*. In white ash (*Fraxinus*), the tetraploid *F. smallii* and hexaploids such as *F. biltmoreana* and *F. profunda* are hypothesized to have allopolyploid origins (Miller, 1955; Nesom, 2010; Santamour, 1962).

Early efforts producing artificial polyploids in lilac were reported to be successful. In the middle of the 20th century, Karl Sax produced colchicine-induced autopolyploids of *S. vulgaris* at the Arnold Arboretum (Fiala and Vrugtman, 2008). Fiala reportedly produced tetraploid forms of *S. julianae*, *S. komarowii*, *S. ×prestoniae*, *S. wolfii*, *S. yunnanensis*, *S. vulgaris*, *S. oblata* and *S. ×hyacinthiflora* (Fiala and Vrugtman, 2008).

Despite these previous reports of induced polyploidy, no cytological evidence exists to support these claims. Lilacs have been bred for centuries, yet polyploid lilac breeding remains a largely unexplored field (Fiala and Vrugtman, 2008). Few modern studies have confirmed successful induction of autopolyploid lilacs. Rose et

al. (2000) created mixoploid and tetraploid lilacs from colchicine-treated cuttings of an interseries hybrid, *S. vulgaris* x *S. pinnatifolia*. Rothleutner (2014) recovered diploids, mixoploids, tetraploids, and octaploids from oryzalin-treated seedlings of *S. reticulata* cultivars. Both Rose et al. (2000) and Rothleutner (2014) used flow cytometry to confirm autopolyploids. In many crops, hybridization between tetraploid and diploid populations has been useful for creating sterile triploid progeny due to meiotic irregularities. Where some fertility exists in triploids, they can provide an important bridge in wide crosses and their range of gametes can be used in the production of high copy number polyploids such as tetraploids, pentaploids, and hexaploids (Wang et al., 2010). Aneuploid progeny have been produced in other woody plants through diploid-triploid hybridization including pears (*Pyrus*; Phillips et al., 2016), elms (*Ulmus*; Santamour, 1971), and poplar (*Populus*; Wang et al., 2010).

The purpose of this study was to explore genome size, ploidy variation, and presence of unreduced gametes in a diverse collection of lilacs including representative species and cultivars from five lilac series and interploid hybrids in series *Syringa*.

Methods and Materials

Plant Material. Lilac taxa were acquired from gardens, arboreta, and nurseries. Representative taxa were obtained from five of the six series within genus *Syringa* including *Syringa, Pubescentes, Villosae, Ligustrina,* and *Pinnatifoliae.* Series *Ligustrae,* which includes genus *Ligustrum* nested within genus *Syringa* (Li et

al., 2012), was not included. Included in our study were 54 total taxa including species, cultivars, and hybrids (Table 3.2). Species and subspecies designations are based on current taxonomy (Chen et al., 2009; Li et al., 2012). In lilac, cultivar or trademark names are rarely interchangeable with only one becoming the market name that commonly identifies a cultivar. As a reference, cultivar and trademark names are reported (Table 3.2), but for simplicity, only market names (cultivar or trademark) are used hereafter. A subset of hybrids was created among selected parent taxa to investigate seedling genome size variation in the following parent cytotype combinations: 3*x* x 2*x*, 2*x* x 3*x*, and 2*x* x 2*x*.

Flow Cytometry. Flow cytometry was used to assess holoploid (2C) genome size (relative to an internal standard) for each individual taxon in the lilac collection at Oregon State University. References to genome size and ploidy follow the terminology proposed by Greilhuber et al., (2005). For each taxon, three vegetative buds or three young, fully expanded leaves were collected to represent a random sample of nuclei. Included with each taxon was a leaf sample of the internal standard of known genome size, *Pisum sativum* 'Ctirad' (2C = 8.76 pg) (Bai et al., 2012; Greilhuber et al., 2007). Each sample was prepared by co-chopping 1-2 cm^2 of tissue from both lilac and an internal standard (*P. sativum* 'Ctirad') with a razor in a polystyrene petri dish containing 400 µL of nuclei extraction buffer solution (Cystain Ultraviolet Precise P Nuclei Extraction Buffer; Sysmex, Görlitz, Germany). Buffer containing chopped leaf tissue was passed through a 30-µm gauze filter (Partec Celltrics, Münster, Germany) into a 3.5-mL plastic tube (Sarstedt Ag & Co.; Nümbrecht, Germany). Next, 1.6 mL of fluorochrome stain (DAPI; 4',6-diamidino-

2-phenylindole) was added to the nuclei suspension (Cystain ultraviolet Precise P Staining Buffer; Partec). All samples were analyzed using a flow cytometer (CyFlow Ploidy Analyzer; Partec). A minimum of 3000 nuclei were analyzed per sample with average coefficient of variation (CV) for each fluorescence histogram under ten. Relative 2C genome size was calculated as:

$$2C = \text{DNA content of standard} \times \frac{\text{mean fluorescence value of sample}}{\text{mean fluorescence value of standard}} \quad .$$

Monoploid (1C*x*) genome size was calculated using ploidy determined using root tip microscopy (described below) as:

$$1Cx = \frac{2C}{\text{ploidy}} \quad .$$

Estimations of chromosome numbers in aneuploid seedlings were based on a genome size estimate of a single, theoretical chromosome (0.061 pg) calculated as:

$$\text{Genome size of a single chromosome} = \frac{\text{Polyploid parent (2C pg) - Diploid parent (2C pg)}}{\text{difference in chromosome number}}$$

Genome size variation in parent taxa and progeny resulting from different cytotype combinations were investigated using flow cytometry. Histogram figures from flow cytometry (.fcs files) were produced using open-source Cytospec software from Purdue University Cytometry Laboratories (PUCL, 2014).

Chromosome counts. Chromosome counts were performed on several taxa representing four series: *Syringa, Pubescentes*, *Villosae,* and *Ligustrina*. An

improved protocol for preparing root tips for chromosome counts (Lattier et al., 2017) was followed for lilac, with lilac root tips digested by enzyme for 2 to 3 h. Chromosomes were visualized (Axio imager.A1, Zeiss, Thornwood, NY) and imaged (AxioCam 105 Color, Zeiss) at different focal distances and layered to increase resolution for each photomicrograph. Focus-stacking was performed using the Auto Blend feature in Photoshop CC 2015.5.1 (Adobe Systems; San Jose, CA). A minimum of 15 resolved cells were investigated per taxa.

Pollen Cytology. From a previous study on cross-compatibility (Lattier and Contreras, 2017), a single seedling from an interploid cross was discovered to have a larger genome size than either parent suggesting that unreduced gametes were present in one parent. Therefore, both parents (*S. vulgaris* 'President Grévy' and *S. vulgaris* 'Sensation') as well as two other randomly selected taxa (*S. vulgaris* 'Ludwig Spaeth' and *S. vulgaris* 'Miss Ellen Willmott') were screened for unreduced pollen grains. At anthesis, fresh flowers were collected from each plant, and pollen was dusted onto microscope slides. Three slides were prepared and measured for each cultivar. To each slide, a single drop of 2% acetocarmine was added and then a cover slip was added. All slides were screened for stained pollen grains at a magnification of ×100 on a light microscope (Axio imager.A1, Zeiss). Single fields of view were randomly captured (AxioCam 105 Color, Zeiss) across each microscope slide and all stained pollen grains were measured using the line measurement tool in AxioVision SE64 4.9.1 (Zeiss). A total of 5381 pollen grains were measured in the four cultivars of *S. vulgaris*. Figures of reduced and unreduced pollen grains were focus-stacked to

increase resolution using the Auto Blend feature of Photoshop CC 2015.5.1 (Adobe Systems). To estimate unreduced pollen grains, the following equation was used:

$$\text{Volume of a sphere} = \frac{4}{3}\pi r^3 .$$

As the volume of a sphere (pollen grain) doubles, the diameter increases by 26%. Therefore, any pollen grains with a diameter greater than 26% of the average for each taxa was scored as an unreduced pollen grain. Percent unreduced pollen grains were calculated as:

$$\text{Percent unreduced pollen} = \frac{\text{unreduced pollen grains}}{\text{total pollen grains}} \times 100 .$$

Statistical Analysis. Statistical analyses were performed using SAS Studio, Version 3.6 (SAS Institute, Cary, NC). Monoploid genome sizes were analyzed with PROC GLM. Mean genome size averages for each individual taxon were separated using Tukey's honestly significant difference test ($\alpha = 0.05$). Genome size averages for each series were generated from an average of individual taxa means. Least squares means for each series were separated using a Tukey-Kramer test for unequal sample sizes ($\alpha = 0.05$). Least squares means were also separated for pollen diameter measurements of four cultivars of *S. vulgaris* using a Tukey-Kramer test for unequal sample sizes ($\alpha = 0.05$).

Results and Discussion

Genome Sizes. Holoploid 2C genome sizes ranged from 2.64 ± 0.08 pg in *S.* ×*laciniata* to 4.94 ± 0.06 pg in *S. vulgaris* 'Aucubaefolia'. All 2C relative genome sizes were larger than the two previously reported genome sizes of European lilacs (Olszewska and Osiecka, 1984; Siljak-Yakovlev et al., 2010). Previous reports have shown similar variation due to different binding properties of fluorochrome stains (Lattier, 2016; Parris et al., 2010). Only three taxa of *S. vulgaris*, nested within series *Syringa*, had a 2C relative genome size larger than 4.00 pg, including *S. vulgaris* 'Aucubaefolia' (4.94 ± 0.06 pg), *S. vulgaris* 'Agincourt Beauty' (4.90 ± 0.03 pg), and *S. vulgaris* 'President Grévy' (4.85 ± 0.00 pg). Chromosome counts of *S. vulgaris* 'Aucubaefolia' revealed this group to be triploids (Fig. 3.1). The presence of triploids in our collection supports early reports of polyploid induction experiments and interploid hybridization (Fiala and Vrugtman, 2008) but surprisingly, no tetraploids were observed. All other root tip cells investigated were diploid, including *S.* ×*hyacinthiflora* 'Maiden's Blush', *S.* ×*hyacinthiflora* 'Old Glory', *S.* ×*prestoniae* 'Miss Canada', *S. reticulata,* and *S. pubescens* Bloomerang® Purple (Fig. 3.1). Chromosome counts in the current study provided no evidence for base chromosome number other than $x = 23$ (Fig. 3.1), in contrast to previous reports that varied from $x = 22$ to 24 (Darlington and Wylie, 1956).

Significant differences were found among taxa for 1C*x* genome size ($P < 0.0001$). Values ranged from 1.32 ± 0.04 pg in *S.* ×*laciniata* to 1.78 ± 0.05 pg in *S.* ×*hyacinthiflora* 'Old Glory' (Table 3.3). Series *Syringa* had a significantly larger average 1C*x* genome size (1.68 ± 0.02 pg) than the other four series investigated

(Table 3.3). There were no significant differences among series *Pubescentes* (1.47 ± 0.01 pg), *Villosae* (1.55 ± 0.02 pg), *Ligustrina* (1.49 ± 0.05 pg), and *Pinnatifoliae* (1.52 ± 0.02 pg) (Table 3.3).

Within series *Syringa*, *S.* ×*laciniata* had a significantly smaller genome size compared to other tested taxa in series *Syringa*. No reports exist on the pedigree of *S.* ×*laciniata* (Table 3.1), although Fiala and Vrugtman (2008) hypothesize it to from a cross of the Afghan lilac, *S. protolaciniata*, and another unknown parent. *Syringa* ×*laciniata* has a heavily dissected leaf, much like *S. pinnatifolia*, while *S. protolaciniata* produces heterophyllous leaves with margins varying from lobed to entire (Fiala and Vrugtman, 2008; Green, 1995). In addition, the only other heavily dissected lilac, *S. pinnatifolia*, has also proven the only species successfully used in interseries crosses (Pringle, 1981). If *S.* ×*laciniata* is from an interseries hybridization, then aneuploidy concomitant with wide hybridization could explain the significant reduction in genome size compared to other taxa in series *Syringa*. Further chromosome counts need to be performed on this hybrid.

The majority of 1C*x* genome sizes within series *Syringa* were above 1.60 pg (Table 3.3). Although *S. oblata* is native to Asia and *S. vulgaris* is native to southeastern Europe, their different geographical origins are not reflected in significant genome size variation. Wild-type *S. oblata* and the white-flowered, *S. oblata* var. *alba*, both had a 1C*x* genome size of 1.73 ± 0.03 pg (Table 3.3). The smallest and largest monoploid genome sizes in *S. vulgaris* were from two white, double-flowered taxa, *S. vulgaris* 'Miss Ellen Willmott' (1.61 ± 0.01 pg) and *S. vulgaris* 'Madame Lemoine' (1.76 ± 0.05 pg) (Table 3.3). Taxa representing hybrids

between *S. oblata* and *S. vulgaris* had a monoploid genome size range from *S.* ×*hyacinthiflora* 'Betsy Ross' (1.70 ± 0.02 pg) to *S.* ×*hyacinthiflora* 'Old Glory' (1.78 ± 0.05 pg), however, there were no differences among the four hybrid cultivars included (Table 3.3). One additional interspecific hybrid, *S.* ×*chinensis* (1.74 ± 0.07 pg), representing a cross between *S. protolaciniata* and *S. vulgaris* was found to have a similar 1C*x* genome size to *S. vulgaris* (Table 3.3).

Within series *Pubescentes*, the majority of 1C*x* genome sizes were below 1.50 pg and there were no significant differences among the twelve taxa included. The smallest genome size was *S. pubescens* Rhythm & Bloom® (1.43 ± 0.01 pg), whereas the largest was in *S. pubescens* ssp. *patula* 'Miss Kim' (1.54 ± 0.01 pg). Within series *Villosae*, the majority of 1C*x* genome sizes were above 1.50 pg, and ranged from *S. tigerstedtii* (1.38 ± 0.01 pg) to *S. villosa* 'Aurea' (1.62 ± 0.03 pg) (Table 3.3). *Syringa villosa* exhibited a 1C*x* genome size similar to cultivars of *S.* ×*prestoniae*, which has *S. villosa* along with *S. komarowii* in its pedigree (Table 3.1). *Syringa tigerstedtii* had a significantly smaller 1C*x* genome size compared to all other taxa except *S.* ×*prestoniae* 'Donald Wyman' (1.50 ± 0.00 pg) and 'Redwine' (1.53 ± 0.02 pg) (Table 3.3). However, no other significant differences were found throughout series *Villosae*, even though this diverse series was the most species-rich in the collection.

Within the tree lilacs (series *Ligustrina*) only two species, *S. reticulata* and *S. pekinensis*, and few cultivars exist. Green and Chang (1995) previously reported only one species, *S. reticulata*, with other species circumscribed to the rank of subspecies. The 1C*x* genome sizes of *S. pekinensis* China Snow® and Summer Charm® were not

significantly different. However, a significant difference was detected between *S. pekinensis* China Snow® (1.41 ± 0.02 pg) and *S. reticulata* (1.59 ± 0.03 pg) (Table 3.3). Within the monotypic series *Pinnatifoliae, S. pinnatifolia* var. *alashanensis* had a 1C*x* genome size of 1.52 ± 0.02 (Table 3.3).

Hybrid genome sizes. Based on genome size estimates of parent taxa, hybrids from a previous cross-compatibility study on lilacs (Lattier and Contreras, 2017) were evaluated for genome size variation. As a seed parent, more than 800 flowers of the triploid *S. vulgaris* 'President Grévy' were pollinated in intraspecific crosses with four different diploids: *S. vulgaris* Tiny Dancer™, *S. vulgaris* 'Angel White', *S. vulgaris* 'President Lincoln', and *S. vulgaris* 'Sensation' (Lattier and Contreras, 2017). Of the four pollen parents, only crosses with the picotee-flowered *S. vulgaris* 'Sensation' produced seed. From 240 pollinations, 107 seeds were obtained; however, only one seed germinated and produced a viable seedling (H2013-150-01). Flow cytometry revealed H2013-150-01 to be a polyploid/aneuploid with a 2C relative genome size of 5.65 ± 0.02 pg (Table 3.4). This genome size was significantly larger than any other seedling and is currently the largest reported in any lilac (Table 3.4) but is lower than expected for a tetraploid. In addition, the genome size of H2013-150-01 was larger than both its seed parent (*S. vulgaris* 'President Grévy' 2C = 4.85 ± 0.00 pg) and its pollen parent (*S. vulgaris* 'Sensation' 2C = 3.33 ± 0.04 pg). In a combined analysis on the flow cytometer, histograms for each parent as well as the hybrid were clearly distinguishable (Fig. 3.2).

Other studies have yielded similar results in crosses between diploids and triploids. Seedlings with genomes sizes surpassing their parents have been reported

for diploid-triploid crosses in other woody plants including pears (Phillips et al., 2016), elms (Santamour, 1971), and poplar (Harder et al., 1976; Wang et al., 2010). Similar results have been found in herbaceous taxa including *Miscanthus sinensis* (Rounsaville et al., 2011), black-eyed-susans (*Rudbeckia*; Palmer et al., 2009), lilies (*Lilium*; Lim et al., 2003; Marasek-Ciolakowska et al., 2014), bananas (*Musa*; Osuji et al., 1997), and cucumbers (*Cucumis sativus*; Diao et al., 2009). The majority of these studies proposed sexual polyploidization via the union of unreduced gametes from one or both parents as the likely cause of the large seedling genomes. In the current study, H2013-150-01 resulted from an unreduced gamete from the diploid *S. vulgaris* 'Sensation' (discussed below) and an aneuploid gamete from the triploid *S. vulgaris* 'President Grévy' that was 0.11 pg below the expected haploid (1.5*x*) value. However, *S. vulgaris* 'Sensation' used in 2*x* x 2*x* reciprocal crosses with *S. vulgaris* Tiny Dancer™ failed to contribute unreduced gametes to produce triploid seedlings (Table 3.4).

As a pollen parent, the triploid *S. vulgaris* 'President Grévy' was used in crosses with *S. vulgaris* Blue Skies®, *S. vulgaris* 'President Lincoln', and *S. vulgaris* 'Sensation' totaling 459 pollinations (Lattier and Contreras, 2017). Crosses with *S. vulgaris* Blue Skies® produced the only viable seed with 12 seedlings recovered from 135 pollinations (Lattier and Contreras, 2017). With the exception of one seedling (H2014-033-11), all hybrid seedlings varied in 2C genome size between the two parents, *S. vulgaris* Blue Skies® (3.44 ± 0.03 pg) and *S. vulgaris* 'President Grévy' (4.85 ± 0.00 pg) (Table 3.4). Relative 2C genome sizes of seedlings varied

significantly from 3.41 ± 0.03 pg (H2014-033-11) to 4.35 ± 0.03 pg (H2014-033-01) (Table 3.4), the majority representing aneuploid genome sizes.

Estimations of chromosome numbers across aneuploid populations can be performed with knowledge of holoploid 2C genome sizes. Previous studies have produced models based on a holoploid genome size of a theoretical average, single chromosome based on parent genome sizes and chromosome counts. Although some estimates of aneuploid chromosomes have been based solely on hypothetical chromosome size (Palmer et al., 2009), several studies have tested this model with root squashes and found most of their predictions to be concurrent with the true chromosome number or accurate within two to three chromosomes in primroses (*Primula*; Hayashi et al., 2009), lilies (Lim et al., 2003) and heathers (*Calluna*; Behrend et al., 2015). Considering these previous studies and the relatively uniform chromosome size observed in lilac (Fig. 3.1), a simple linear model was used to predict chromosome number in the 2*x* x 3*x* aneuploid population (Fig. 3.3).

Based on a linear model with an average chromosome size of 0.06 pg (Fig. 3.3), the chromosome numbers in our aneuploid seedlings from *S. vulgaris* Blue Skies® x *S. vulgaris* 'President Grévy' varied from 46 to 61 with an average of 54.3 ± 1.4 chromosomes. Chromosome numbers of triploid gametes can be deduced by subtracting the euploid chromosome number from the seedlings somatic chromosome numbers (Iorizzo et al., 2012). Assuming that *S. vulgaris* Blue Skies® consistently contributed haploid gametes with 23 chromosomes, *S. vulgaris* 'President Grévy' produced a range of aneuploid pollen from 23 to 38 chromosomes to progeny from this cross. Previous research in other crops has shown that triploids produce a higher

percentage unreduced and/or aneuploid gametes than their diploid or tetraploid counterparts (Burton and Husband, 2001; Herben et al., 2016; Ramsey and Schemske, 1998). Viable aneuploid gametes have been described in plants such as cucumber (Diao et al., 2009), oilseed rape (*Brassica*; Lu and Kato, 2001), and tulips (*Tulipa*; Marasek-Ciolakowska et al., 2014), yet other plants only tolerate euploid gametes as in highbush blueberries (*Vaccinium corymbosum*; Vorsa and Ballington, 1991). The resulting aneuploids from the 2*x* x 3*x* lilac crosses were skewed slightly to the diploid cytotype compared to a theoretical bimodal distribution with an average of 57.5 chromosomes (Fig. 3.3). In the 3*x* x 2*x* cross, *S. vulgaris* 'President Grévy' x *S. vulgaris* 'Sensation', the triploid parent contributed 39 chromosomes (2*n* – 7) as a seed parent. It is unclear if this slightly higher contribution from the triploid is due to combining with an unreduced gamete, the direction of the cross, or chance. Brandham (1982) reported a greater prevalence of aneuploidy over the range between diploid and triploid when the latter are females – presumably associated with endosperm balance number (discussed below). However, with only a single seedling it is impossible to draw conclusions.

Though our seedling cytotypes varied from a random distribution of aneuploid cytotypes, lilac aneuploid segregation conflicts with the limited number of similar studies on 2*x* x 3*x* crosses by being less concentrated at either euploid level (diploid or triploid). In lilies, these crosses resulted in all triploid or near-triploid seedlings derived from viable 2*n* gametes from triploid male parents (Marasek-Ciolakowska et al., 2014). In tulips, 2*x* x 3*x* crosses yielded a majority of diploid and near-diploid progeny with a small percent of near-triploids (Mizuochi et al., 2009). This same

study found that the reciprocal cross in tulips yielded a binomial distribution of aneuploids, with the female triploid parent producing a wide range of fertile aneuploids (Mizuochi et al., 2009). Similar to lilies, 2*x* x 3*x* crosses in chives (Levan, 1936) and cucumbers (Diao et al., 2009) resulted in diploids or near-diploids with a small percent of near-triploids; the reciprocal crosses yielded a wider range of aneuploids. Brandham (1982) reviewed a number of studies on interploid crosses and found that with very few exceptions the triploid parent generally produced gametes that were either haploid or diploid based on the ploidy of the other parent. Poplar was a notable exception from other examples given, namely a considerably higher chromosome number than other taxa discussed. *Populus* (*x* = 19) is similar to *Syringa* (*x* = 23) in chromosome number and both are almost certainly of polyploid origin. This high chromosome number indicates that there is redundancy present that likely allows survival of aneuploid gametes produced by triploids that are inviable in taxa with fewer base chromosomes.

Embryo and endosperm cytotypes often play a role in seedling cytotype segregation, which has been studied at length in diploid and triploid crosses of potatoes (*Solanum*). A 2:1 maternal to paternal endosperm balance ratio must be maintained for successful hybridization in potato, preventing 2*x* x 3*x* crosses and yielding progeny from 3*x* x 2*x* that are skewed to near-triploid cytotypes (Carputo, 1999; Iorizzo et al., 2012). The origin of *S. vulgaris* 'President Grévy' could play a role in its fertility as a triploid parent. Allotriploids are rarely used in breeding due to their difficulty in chromosome pairing during meiosis; however, autotriploids can overcome problems with meiotic pairing to produce haploid to triploid gametes

(Brandham, 1982; Hayashi et al., 2009; Kato et al., 2001). The history of wide hybridization and polyploid induction in lilac leaves the question open to the origins of triploid cytotypes. In addition, it remains unclear if meiotic abnormalities in gamete formation, preferential fertilization, or preferential embryo/endosperm survival skewed the distribution of aneuploid cytotypes. Our results may simply be due to the small sampling population of aneuploid seedlings resulting from the 2*x* x 3*x* and 3*x* x 2*x* crosses.

Despite the numerous pollinations and few resulting seedlings, all aneuploid lilacs appear to be healthy and vigorous after their first 2 years of growth (J.D. Lattier, personal observation). This conflicts with some studies which reported aneuploid seedlings from diploid-triploid crosses were non-viable past initial germination exhibiting abnormal, stunted growth (Behrend et al., 2015; Osuji et al., 1997) and sometimes reverting to euploids after 1+ year (Behnrend et al., 2015). Though our aneuploids appear to grow as vigorously as their diploid counterparts, female fertility and pollen viability in the aneuploid population has not been investigated as the plants have yet to reach maturity during this study. However, flow cytometry was performed on the aneuploid population more than 2 years after germination. While it cannot be assured that this aneuploid series will not stabilize at a euploid level (diploid or triploid), the fact that these plants have maintained aneuploid chromosome compliments for more than 2 years suggests they may be stable.

Unreduced pollen. Stained pollen grains from four taxa of *S. vulgaris* were scored as viable and were measured for variability in diameter. Unstained pollen

grains were negligible in all taxa, and pollen germination was not investigated. There were significant differences among taxa for pollen diameter ($P < 0.0001$) and every pairwise comparison between taxa was significant ($P < 0.01$). The largest average pollen grains were detected in *S. vulgaris* 'Sensation' (35.74 ± 0.16 μm), a measure likely overinflated by the presence of 8.5% unreduced pollen grains identified due to their increased volume (Fig. 3.4). This is the first report of unreduced (2*n*) pollen in lilac, but is not the first report in the Oleaceae. B-chromosomes and unreduced pollen have been reported in cultivars of olive (Sheidai et al., 2008). *Syringa vulgaris* 'Sensation' was also the only diploid observed to produce unreduced pollen, as *S. vulgaris* 'Ludwig Spaeth' and *S. vulgaris* 'Miss Ellen Willmott' produced only 1*n* pollen (Fig. 3.4).

Due to its low fertility in crosses with the triploid seed parent *S. vulgaris* 'President Grévy' and the presence of an inflated genome size in the single viable seedling recovered (H2013-150-01), an unreduced pollen grain from *S. vulgaris* 'Sensation' likely contributed to the production of this single polyploid/aneuploid progeny. It remains unclear whether the rare picotee flower mutation is in some way related to the production of unreduced pollen, or if other diploids of *S. vulgaris* that were not included in the current study produce unreduced pollen at a similar rate as *S. vulgaris* 'Sensation'. Since an unreduced pollen grain resulted in the only viable seedling between these two cultivars, this may indicate the utility of increased ploidy level for improving cross-compatibility in lilac.

Pollen from the double-flowering, triploid *S. vulgaris* 'President Grévy' proved difficult to obtain as many flowers simply did not produce viable anthers.

Unreduced pollen grains (0.6%) were detected in *S. vulgaris* ‘President Grévy’ out of 1689 grains measured. Though at a much lower percentage, *S. vulgaris* ‘President Grévy’ was the only parent besides *S. vulgaris* ‘Sensation’ to produce unreduced pollen in the current study, indicating some level of meiotic irregularities. Triploids are more likely to undergo irregularities during meiosis such as irregular chromosome pairing, supernumerary B chromosomes, laggard chromosomes, chromatin bridges, cytomixis, and out of plate chromosomes during metaphase I (Farco and Dematteis, 2014; Lavia et al., 2011). Triploids and resulting aneuploid progeny may prove to be sterile, yielding cultivars with reduced weediness and extended bloom times. Irregular meiosis during microspore development likely contributed to the poor performance of *S. vulgaris* ‘President Grévy’ as a seed parent and the subsequent aneuploidy seen in its viable seedlings (Fig. 3.3). Based on its aneuploid offspring when used as a male parent, *S. vulgaris* ‘President Grévy’ likely produces a range of aneuploid pollen. The average pollen grain diameter of *S. vulgaris* ‘President Grévy’ (35.28 ± 0.07 μm) was significantly larger than the two diploids that exhibited normal meiosis, *S. vulgaris* ‘Ludwig Spaeth’ (33.96 ± 0.05 μm) and *S. vulgaris* ‘Miss Ellen Willmott’ (34.32 ± 0.06 μm) from which no unreduced gametes were observed in a combined 2866 pollen grains (Fig. 3.4).

This study provides valuable information for future lilac breeding and informs a previous study on cross-compatibility among elite cultivars of lilac (Lattier and Contreras, 2017). In addition, this study contributes genome size and ploidy information to the growing database of angiosperm genome sizes, recommended by Galbraith et al. (2011). The discovery of three triploid lilacs, *S. vulgaris*

‘Aucubaefolia’, *S. vulgaris* ‘Agincourt Beauty’, and *S. vulgaris* ‘President Grévy’ lends evidence to previous reports of artificial tetraploid development and subsequent hybridization (Fiala and Vrugtman, 2008). However, no tetraploids were discovered among the research population. The discovery of high levels of aneuploidy in interploid hybrids indicates meiotic irregularities in pollen development of polyploid lilacs. Further cytological studies of pollen mother cells and meiotic analyses could contribute to understanding the complexities within developing gametes of taxa in the heavily hybridized series *Syringa*.

The development of an aneuploid series in 2*x* x 3*x* crosses provides an avenue to develop a model for cytotype prediction in seedlings of interploid lilac hybrids. Future efforts to confirm initial predictions of aneuploid chromosome numbers will include chromosome counts on parent taxa, chromosome counts on a subset of aneuploids, and re-pollination of the parent genotypes to increase the number of seedlings in the aneuploid population. Further, aneuploids can be highly variable in morphology, including reduced vigor, and can have greatly reduced fertility. This may be a detriment in breeding most crops, but could be an avenue for ornamental breeders to recover more compact, longer-blooming, sterile cultivars. As the aneuploid population matures, plants will be compared for differences in gross morphology and flowers will be compared for pollen viability and female fertility. Reanalyzing the genome sizes of this population will be necessary over subsequent years in light of previous reports of euploidization of woody aneuploids (Behrend et al., 2015).

The discovery of unreduced pollen in *S. vulgaris* ‘Sensation’, and subsequent production of a seedling from a 3*x* x 2*x* cross with a larger genome than either parent, indicate that unreduced gametes or polyploidy may contribute to cross-compatibility in wide hybridization of lilac. Future work using high-throughput pollen screening by flow cytometry may reveal other cultivars with high levels of unreduced gametes. Wide hybridization with polyploids may reduce the impact of chromosome loss, which has been reported in previous cytological studies on lilac (Taylor, 1945) and the smallest genome recorded in the current study was a dissected-leaved, interspecific hybrid, *S.* ×*laciniata*. Identifying parents with unreduced pollen or generating autopolyploids in each lilac series may prove a valuable method for recovering viable progeny from wide hybridization in lilac. Interseries hybrids continue to be the most elusive quarry for lilac breeders, with only the pinnately compound *S. pinnatifolia* in the monophyletic series *Pinnatifoliae* proven a successful parent in crosses with *S. oblata* var. *giraldii*, *S. vulgaris*, *S.* ×*laciniata*, and *S.* ×*hyacinthiflora* (Pringle, 1981). Using *S. pinnatifolia*, as well as induced autopolyploids of cultivars proven to produce fruit and seed in interseries crosses (Lattier and Contreras, 2017) may spark a renaissance in the storied history of lilac breeding.

Literature Cited

Bai, C., W.S. Alverson, A. Follansbee, and D.M. Waller. 2012. New reports of nuclear DNA content for 407 vascular plant taxa from the United States. Ann. Bot. 110:1623-1629.

Behrend, A., A. Gluschak, A. Przybyla, and A. Hohe. 2015. Interploid crosses in heather (*Calluna vulgaris*). Scientia Hort. 181:162-167.

Bennett, M.D. and I.J. Leitch. 2011. Nuclear DNA amounts in angiosperms: targets, trends and tomorrow. Ann. Bot. 107:467-590.

Brandham, P.E. 1982. Inter-embryo competition in the progeny of autotriploid Aloineae (Liliaceae). Genetica 59:29-42.

Burton, T.L. and B.C. Husband. 2001. Fecundity and offspring ploidy in matings among diploid, triploid and tetraploid *Chamerion angustifolium* (Onagraceae): Consequences for tetraploid establishment. Heredity 87:573–582.

Carputo, D. 1999. Post-zygotic gametic selection due to endosperm balance number explains unusual chromosome numbers of 3x x 2x progeny in *Solanum*. Sexual Plant Reproduction 12:27-31.

Chen, J.-T., Z.-S. Zhang, and D.-Y. Hong. 2009. A taxonomic revision of the *Syringa pubescens* complex (Oleaceae). Ann. Missouri Bot. Garden 96:237-250.

Chen, Z.J. and Z. Ni. 2006. Mechanisms of genomic rearrangements and gene expression changes in plant polyploids. Bioessays 28:240-252.

Contreras, R.N., M. Friddle, and J.D. Lattier. 2013. Relative fertility and ploidy levels of selected rose-of-sharon cultivars. Proc. 58th Ann. Southern Nursery Assn. Res. Conf. 58:232-236.

Contreras, R.N., T.G. Ranney, and S.P. Tallury. 2007. Reproductive behavior of diploid and allotetraploid *Rhododendron* L. 'Fragrant Affinity'. HortScience 42:31-34.

Darlington, C.D. and A.P. Wylie. 1956. Chromosome atlas of flowering plants. Macmillan, New York, NY.

Diao, W.-P., S.-Y. Bao, B. Jiang, L. Cui, and J.-F. Chen. 2009. Primary trisomics obtained from autotriploid by diploid reciprocal crosses in cucumber. Sexual Plant Reproduction 22:45-51.

Farco, G.E. and M. Dematteis. 2014. Meiotic behavior and pollen fertility in trioploid and tetraploid natural populations of *Campuloclinum macrocephalum* (Eupatorieae, Asteraceae). Plant Systematics Evolution 300:1843-1852.

Fiala, J.L. and F. Vrugtman. 2008. Lilac: A gardener's encyclopedia. 2nd ed. Timber Press, Portland, OR.

Galbraith, D.W., J.L. Bennetzen, E.A. Kellogg, J.C. Pires, and P.S. Soltis. 2011. The genomes of all angiosperms: A call for a coordinated global census. J. Bot. 2011:1-10.

Green, P.S. 1995. Proposal to reject the name *Syringa buxifolia* Nakai (Oleaceae). Taxon 44:636.

Green, P.S. and M.C. Chang. 1995. Some taxonomic changes in *Syringa* L. (Oleaceae), including a revision of series *Pubescentes*. Novon 5:329-333.

Greilhuber, J. 1998. Intraspecific variation in genome size: A critical reassessment. Ann. Bot. 82:27-35.

Greilhuber, J., E.M. Temsch, and J.C.M. Loureiro. 2007. Nuclear DNA content measurement, p. 67-101. In: J. Doležel, J. Greilhuber, and J. Suda (eds.). Flow cytometry with plant cells: Analysis of genes, chromosomes and genomes. Wiley, Weinheim, Germany.

Greilhuber, J., J. Doležel, M.A. Lysák, and M.D. Bennett. 2005. The origin, evolution and proposed stabilization of the terms 'genome size' and 'C-value' to describe nuclear DNA contents. Ann. Bot. 95:255–260.

Harder, M.L., S. Verhagen, L. Winton, and D.W. Einspahr. 1976. Tetraploid aspen production using unreduced pollen from triploid males. For. Sci. 22:329-330.

Harlan, J.R. and J.M.J. deWet. 1975. On Ö. Winge and a prayer: The origins of polyploidy. Bot. Rev. 41:361-390.

Hayashi, M., J. Kato, H. Ohashi, and M. Mii. 2009. Unreduced 3*x* gamete formation of allotriploid hybrid derived from the cross of *Primula denticulata* (4*x*) x *P. rosea* (2*x*) as a causal factor for producing pentaploid hybrids in the backcross with pollen of tetraploid *P. denticulata*. Euphytica 169:123-131.

Herben, T., P. Trávníček, and J. Chrtek. 2016. Reduced and unreduced gametes combine almost freely in a multiploidy system. Perspectives Plant Ecol. Evolution Systematics 18:15-22.

Iorizzo, M., R. Aversano, J.M. Bradeen, L. Frusciante, and D. Carputo. 2012. Fertilization fitness and offspring ploidy in 3x x 2x matings in potato. Plant Biosystems 146:317-321.

Kato, J., R. Ishikawa, and M. Mii. 2001. Different genetic combinations in inter-section hybrids obtained from the crosses between *Primula sieboldii* (section *Cortusoides*) and *P. obconica* (section *Obconicolisteri*) by the embryo rescue technique. Theoretical Appl. Genet. 102:1129-1135.

Kim, K.-J. and R.K. Jansen. 1998. A chloroplast DNA phylogeny of lilacs (*Syringa*, Oleaceae): Plastome groups show strong correlations with crossing groups. Amer. J. Bot. 85:1338-1351.

Lattier, J.D. and R.N. Contreras. 2017. Intraspecific, interspecific, and interseries cross-compatibility in lilac. J. Amer. Soc. Hort. Sci. 142: 279-288.

Lattier, J.D., H. Chen, and R.N. Contreras. 2017. Improved method for enzyme digestion and cytology of woody plant root tips. HortScience (In press).

Lattier, K.S. 2016. Inducing and evaluating phenotypic and cytometric variation in landscape plants: Observations from *Acer*, *Ornithogalum*, and *Penstemon*. Oregon State University, Corvallis, M.S. Thesis.

Lavia, G.I., A.M. Ortiz, G. Robledo, A. Fernández, and G. Seijo. 2011. Origin of triploid *Arachis pintoi* (Leguminosae) by autopolyploidy evidenced by FISH and meiotic behaviour. Ann. Bot. 108:103-111.

Levan, A. 1936. Different results in reciprocal crosses between diploid and triploid *Allium schoenoprasum* L. Nature 138:508.

Li, J., B. Goldman-Huertas, J. DeYoung, and J. Alexander III. 2012. Phylogenetics and diversification of *Syringa* inferred from nuclear and plastid DNA sequences. Castanea 77:82-88.

Li, J., J.H. Alexander, and D. Zhang. 2002. Paraphyletic *Syringa* (Oleaceae): Evidence from sequences of nuclear and ribosomal DNA ITS and ETS regions. Systematic Bot. 27:592-597.

Lim, K.-B., M.S. Ramanna, E. Jacobsen, and J.M. van Tuyl. 2003. Evaluation of BC_2 progenies derived from 3*x*-2*x* and 3*x*-4*x* crosses of *Lilium* hybrids: A GISH analysis. Theoretical Appl. Genet. 106:568-574.

Lu, C. and M. Kato. 2001. Fertilization fitness and relation to chromosome number in interspecific progeny between *Brassica napus* and *B. rapa*: A comparative study using natural and resynthesized *B. napus*. Breeding Sci. 51:73-81.

Marasek-Ciolakowska, A., S. Xie, P. Arens, and J.M. van Tuyl. 2014. Ploidy manipulation and introgression breeding in darwin hybrid tulips. Euphytica 198:389-400.

Miller, G.N. 1955. The genus *Fraxinus*, the ashes, in North America, north of Mexico. Cornell Univ. Agr. Expt. Station Memoirs 335:1-64.

Mizuochi, H., H. Matsuzaki, T. Moue, and K. Okazaki. 2009. Diploid endosperm formation in *Tulipa* spp. and identification of a 1:1 maternal-to-paternal genome ratio in endosperms of *T. gesneriana* L. Sexual Plant Reproduction 22:27-36.

Nesom, G.L. 2010. *Fraxinus biltmoreana* and *Fraxinus smallii* (Oleaceae), forest trees of the eastern United States. Phytoneuron 51:1-30.

Olsen, R.T., T.G. Ranney, and Z. Viloria. 2006. Reproductive behavior of induced allotetraploid ×*Chitalpa* and in vitro embryo culture of polyploid progeny. J. Amer. Soc. Hort. Sci. 131:716-724.

Olszewska, M.J. and R. Osiecka. 1984. Relationship between 2C DNA content, systematic position and level of DNA endoreplication during differentiation of root parenchyma in dicot shrubs and trees. Comparison with herbaceous species. Biochemie und Physiologie der Pflanzen 179:641-657.

Osuji, J.O, D. Vuylsteke, and R. Ortiz. 1997. Ploidy variation in hybrids from interploid 3*x* x 2*x* crosses in *Musa*. Tropicultura 15:37-39.

Palmer, I.E., T.G. Ranney, N.P. Lynch, and R.E. Bir. 2009. Crossability, cytogenetics, and reproductive pathways in *Rudbeckia* subgenus *Rudbeckia*. HortScience 44:44-48.

Parris, J.K., T.G. Ranney, H.T. Knap, and W.V. Baird. 2010. Ploidy levels, relative genome sizes, and base pair composition in *Magnolia*. J. Amer. Soc. Hort. Sci. 135:533-547.

Phillips, W.D., T.G. Ranney, D.H. Touchell, and T.A. Eaker. 2016. Fertility and reproductive pathways of triploid flowering pears (*Pyrus* sp.). HortScience 51:968-971.

Pringle, J.S. 1981. A review of attempted and reported interseries and intergeneric hybridization in *Syringa* (Oleaceae). Baileya 20:49-91.

Purdue University Cytometry Laboratories (PUCL). 2014. http://www.cyto.purdue.edu/Purdue_software

Ramsey, J. and D.W. Schemske. 1998. Pathways, mechanisms, and rates of polyploid formation in flowering plants. Annu. Rev. Ecol. Systematics 29:467-501.

Ranney, T.G. 2006. Polyploidy: from evolution to new plant development. Proc. Intl. Plant Propagators Soc. 56:604-607.

Rose, J.B., J. Kubba, and K.R. Tobutt. 2000. Chromosome doubling in sterile *Syringa vulgaris* x *S. pinnatifolia* hybrids by in vitro culture of nodal explants. Plant Cell Tissue Organ Cult. 63:127-132.

Rothleutner, J.J. 2014. Development of autopolyploid *Syringa reticulata* subsp. *pekinensis* for breeding. Acta Hort. 1055:213-217.

Rounsaville, T.J. and T.G. Ranney. 2010. Ploidy levels and genome sizes of *Berberis* L. and *Mahonia* Nutt. species, hybrids, and cultivars. HortScience 45:1029-1033.

Rounsaville, T.J., D.H. Touchell, and T.G. Ranney. 2011. Fertility and reproductive pathways in diploid and triploid *Miscanthus sinensis*. HortScience 46:1353-1357.

Santamour, Jr., F.S. 1962. The relation between polyploidy and morphology in white and biltmore ashes. Bul. Torrey Bot. Club 89:228-232.

Santamour, Jr., F.S. 1971. A tripoid elm (*Ulmus pumila* x *U. rubra*) and its aneuploid progeny. Bul. Torrey Bot. Club 98:310-314.

Sax, K. 1930. Chromosome number and behavior in the genus *Syringa*. J. Arnold Arboretum 11:7-14.

Shearer, K. and T.G. Ranney. 2013. Ploidy levels and relative genome size of species, hybrids, and cultivars of dogwood (*Cornus* spp.). HortScience 48:825-830.

Sheidai, M., H. Parsian, S. Vaezi-Joze, and Z. Noormohammadi. 2008. Chromosome pairing and chiasma formation in some olive (*Olea europaea* L.) cultivars from Iran. Cytologia 73:269-274.

Siljak-Yakovlev, S., F. Pustahija, E.M. Šolić, F. Bogunić, E. Muratović, N. Bašić, O. Catrice, and S.C. Brown. 2010. Towards a genome size and chromosome number database of Balkan flora: C-Values in 343 taxa with novel values for 242. Advanced Sci. Lett. 3:190-213.

Soltis, P.S. and D.E. Soltis (eds.). 2012. Polyploidy and genome evolution. Springer-Verlag Berlin, Germany.

Stebbins, Jr., G.L. 1940. The significance of polyploidy in plant evolution. Amer. Naturalist 74:54-66.

Taylor, H. 1945. Cyto-taxonomy and phylogeny of the Oleaceae. Brittonia 5:337-367.

Vorsa, N. and J.R. Ballington. 1991. Fertility of triploid highbush blueberry. J. Amer. Soc. Hort. Sci. 116:336-341.

Wallander, E. and V.A. Albert. 2000. Phylogeny and classification of Oleaceae based on *rps16* and *trnL-F* sequence data. Amer. J. Bot. 87:1827-1841.

Wang, J., X. Kang, and Q. Zhu. 2010. Variation in pollen formation and its cytological mechanism in an allotriploid white poplar. Tree Genet. Genomes 6:281-290.

Zonneveld, B.J.M. and G.D. Duncan. 2010. Genome sizes of *Eucomis* L'Hér. (Hyacinthaceae) and a description of the new species *Eucomis grimshawii* G.D. Duncan & Zonneveld. Plant Systematics Evolution 284:99-109.

Zonneveld, B.J.M., I.J. Leitch, and M.D. Bennett. 2005. First nuclear DNA amounts in more than 300 angiosperms. Ann. Bot. 96:229-244.

Tables

Table 3.1. Previously named interspecific hybrids in lilac including their series and pedigree information (Fiala and Vrugtman, 2008).

Interspecific hybrid	♀ parent	♀ series	♂ parent	♂ series
Syringa ×chinensis	*S. protolaciniata*	*Syringa*	*S. vulgaris*	*Syringa*
Syringa ×diversifolia[z]	*S. pinnatifolia*	*Pinnatifoliae*	*S. oblata* ssp. *oblata*	*Syringa*
Syringa ×henryi	*S. josikaea*	*Villosae*	*S. villosa*	*Villosae*
Syringa ×hyacinthiflora	*S. oblata*	*Syringa*	*S. vulgaris*	*Syringa*
Syringa ×josiflexa	*S. josikaea*	*Villosae*	*S. reflexa*	*Villosae*
Syringa ×laciniata	unknown	unknown	unknown	unknown
Syringa ×nanceiana	*S. ×henryi*	*Villosae*	*S. sweginzowii*	*Villosae*
Syringa ×persica	unknown	unknown	unknown	unknown
Syringa ×prestoniae	*S. villosa*	*Villosae*	*S. komarowii*	*Villosae*
Syringa ×swegiflexa	*S. komarowii*	*Villosae*	*S. sweginzowii*	*Villosae*

[z]The single named interspecific hybrid resulting from an interseries cross in lilacs.

Table 3.2. Taxonomic, trademark, accession, and source information for lilac (*Syringa*) source material used in the current study.

Series[z]	Taxon[y]	Cultivar (trademark name)[x]	Accession no.[w]	Source[v]
Syringa	*S. oblata*		09-0058	Arborétum Mlyňany
	S. oblata var. *alba*		09-0059	Arborétum Mlyňany
	S. vulgaris	Agincourt Beauty	13-0036	Briggs Nursery
		Angel White	10-0043	Blue Heron Farm
		Aucubaefolia	13-0039	Briggs Nursery
		Charles Joly	14-0127	Dennis' 7 Dees
		E.J. Gardner	15-0014	Blue Heron Farm
		Miss Ellen Willmott	14-0215	Portland Nursery
		Monore (Blue Skies®)	13-0076	Monrovia
		Katherine Havemeyer	15-0014	Blue Heron Farm
		Krasavitsa Moskvy	13-0043	Briggs Nursery
		Lavender Lady	13-0078	Monrovia
		Ludwig Spaeth	10-0042	Blue Heron Farm
		Madame Lemoine	14-0122	Portland Nursery
		Prairie Petite	13-0035	Briggs Nursery
		President Grévy	10-0040	Blue Heron Farm
		President Lincoln	13-0080	Monrovia
		Primrose	13-0040	Briggs Nursery
		Sensation	13-0081	Monrovia
		Elsdancer (Tiny Dancer™)	13-0001	Heritage Seedlings
	S. ×*hyacinthiflora*	Betsy Ross	13-0034	Briggs Nursery
		Maiden's Blush	14-0123	Dennis' 7 Dees
		Old Glory	13-0085	Monrovia
		Pocahontas	13-0084	Monrovia
	S. ×*chinensis*	Lilac Sunday	13-0041	Briggs Nursery
	S. ×*laciniata*		LS	OSU campus
Pubescentes	*S. meyeri*	Palabin	10-0209	Bailey Nurseries
	S. pubescens	Penda (Bloomerang® Purple)	12-0026	Garland Nursery
		SMSJBP7 (Bloomerang® Dark Purple)	13-0071	Monrovia
		MORjos 060F (Josee™)	10-0039	Blue Heron Farm
		Bailbelle (Tinkerbelle®)	12-0027	Bailey Nurseries
		Bailsugar (Sugar Plum Fairy®)	14-0190	Select Plus
		Colby's Wishing Star	14-0191	Select Plus
		SMSXPM (Scent and Sensibility™)	13-0074	Monrovia
		Red Pixie	16-0013	Forest Farm
		SMSMPRZ1 (Rhythm & Bloom®)	15-0018	Kraemer's Nursery

Table 3.2 (continued). Taxonomic, trademark, accession, and source information for lilac (*Syringa*) source material used in the current study.

Series[z]	Taxon[y]	Cultivar (trademark name)[x]	Accession no.[w]	Source[v]
Pubescentes (continued)				
	S. pubescens ssp. *patula*		13-0072	Monrovia
	S. pubescens ssp. *patula*	Miss Kim	13-0073	Monrovia
Villosae	*S. emodi*		09-0038	Hohenheim Gardens
	S. josikaea		09-0039	Hohenheim Gardens
	S. julianae		09-0057	Arborétum Mlyňany
	S. sweginzowii		11-0021	NBG Dublin
	S. tigerstedtii		09-0040	Hohenheim Gardens
	S. villosa		09-0061	Arborétum Mlyňany
		Aurea	13-0038	Briggs Nursery
	S. wolfii		09-0062	Arborétum Mlyňany
	S. ×prestoniae	Miss Canada	13-0037	Briggs Nursery
		Donald Wyman	13-0086	Monrovia
		Redwine	13-0088	Monrovia
	S. yunnanensis		09-0063	Arborétum Mlyňany
Ligustrina	*S. pekinensis*	Morton (China Snow®)	LS	Carlton Nursery
	S. pekinensis	DTR 124 (Summer Charm®)	LS	Carlton Nursery
	S. reticulata		09-0060	Arborétum Mlyňany
Pinnatifoliae	*S. pinnatifolia* var. *alashanensis*		13-0026	Briggs Nursery

[z]Series designation based on Li et al. (2012).

[y]Individual taxon in *Syringa* based on (Li et al. 2012) and revisions (Chen et al., 2009).

[x]Cultivar and trademark name.

[w]Accession number in research population. LS = non-accessioned leaf samples for flow cytometry.

[v]Container plants, seeds, and leaf samples collected from the following sources: Arborétum Mlyňany, Slepcany, Slovakia; Bailey Nurseries, Yamhill, OR; Blue Heron Farm, Corvallis, OR; Briggs Nursery, Elma, WA; Carlton Plants, Dayton, OR; Dennis' 7 Dees Landscaping & Garden Centers, Portland, OR; Garland Nursery, Corvallis, OR; Heritage Seedlings & Liners, Salem, OR; Hohenheim Gardens, Stuttgart, Germany; Kraemer's Nursery, McMinnville, OR; Monrovia, Dayton, OR; National Botanic Gardens (Dublin), Glasnevin, Ireland; OSU (Oregon State University) Campus, Corvallis, OR; Portland Nursery, Portland, OR; Select Plus International Lilac Nursery, Quebec, Canada.

Table 3.3. Ploidy and relative genome size in lilac (*Syringa*) determined using flow cytometry analysis of DAPI-stained nuclei with *Pisum sativum* 'Ctirad' (8.76 pg/2C) as an internal standard.

Series[z]	1C*x* genome size [mean ± SE (pg)][y]	Taxa[x]	Ploidy[w]	1C*x* genome size [mean ± SE (pg)][v]
Syringa	1.68 ± 0.02 A	*S. oblata*	2*x*	1.73 ± 0.03 A-D
		S. oblata var. *alba*	2*x*	1.73 ± 0.03 A-C
		S. vulgaris 'Agincourt Beauty'	3*x*	1.63 ± 0.01 A-M
		S. vulgaris 'Angel White'	2*x*	1.67 ± 0.05 A-K
		S. vulgaris 'Aucubaefolia'	3*x*[u]	1.65 ± 0.02 A-L
		S. vulgaris Blue Skies®	2*x*	1.72 ± 0.02 A-E
		S. vulgaris 'Charles Joly'	2*x*	1.69 ± 0.02 A-H
		S. vulgaris 'E.J. Gardner'	2*x*	1.66 ± 0.01 A-K
		S. *vulgaris* 'Miss Ellen Willmott'	2*x*	1.61 ± 0.01 B-O
		S. vulgaris 'Katherine Havemeyer'	2*x*	1.71 ± 0.03 A-E
		S. vulgaris 'Krasavitsa Moskvy'	2*x*	1.70 ± 0.00 A-H
		S. vulgaris 'Lavender Lady'	2*x*	1.69 ± 0.02 A-H
		S. vulgaris 'Ludwig Spaeth'	2*x*	1.74 ± 0.03 A-C
		S. vulgaris 'Madame Lemoine'	2*x*	1.76 ± 0.05 A-B
		S. vulgaris 'Prairie Petite'	2*x*	1.69 ± 0.03 A-I
		S. vulgaris 'President Grévy'	3*x*	1.62 ± 0.00 B-O
		S. vulgaris 'President Lincoln'	2*x*	1.73 ± 0.01 A-D
		S. vulgaris 'Primrose'	2*x*	1.68 ± 0.01 A-J
		S. vulgaris 'Sensation'	2*x*	1.67 ± 0.02 A-K
		S. vulgaris Tiny Dancer™	2*x*	1.71 ± 0.02 A-G
		S. ×*hyacinthiflora* 'Betsy Ross'	2*x*	1.70 ± 0.02 A-G
		S. ×*hyacinthiflora* 'Maiden's Blush'	2*x*[u]	1.74 ± 0.07 A-C
		S. ×*hyacinthiflora* 'Old Glory'	2*x*[u]	1.78 ± 0.05 A
		S. ×*hyacinthiflora* 'Pocahontas'	2*x*	1.75 ± 0.02 A-B
		S. ×*chinensis* 'Lilac Sunday'	2*x*	1.74 ± 0.07 A-C
		S. ×*laciniata*	2*x*	1.32 ± 0.04 U
Pubescentes	1.47 ± 0.01 B	*S. meyeri* 'Palabin'	2*x*	1.47 ± 0.03 N-U
		S. pubescens Bloomerang® Purple	2*x*[u]	1.46 ± 0.02 O-U
		S. pubescens Bloomerang® Dark Purple	2*x*	1.49 ± 0.04 L-T
		S. pubescens 'Colby's Wishing Star'	2*x*	1.52 ± 0.02 K-T
		S. pubescens Josee™	2*x*	1.45 ± 0.04 P-U
		S. pubescens 'Red Pixie'	2*x*	1.49 ± 0.01 L-T
		S. pubescens Rhythm & Bloom®	2*x*	1.43 ± 0.01 Q-U
		S. pubescens Scent and Sensibility™	2*x*	1.47 ± 0.00 N-U

Table 3.3 (continued). Ploidy and relative genome size in lilac (*Syringa*) determined using flow cytometry analysis of DAPI-stained nuclei with *Pisum sativum* 'Ctirad' (8.76 pg/2C) as an internal standard.

Series[z]	1C*x* genome size [mean ± SE (pg)][y]	Taxa[x]	Ploidy[w]	1C*x* genome size [mean ± SE (pg)][v]
Pubescentes (continued)				
		S. pubescens Sugar Plum Fairy®	2*x*	1.47 ± 0.03 N-U
		S. pubescens Tinkerbelle®	2*x*	1.40 ± 0.01 S-U
		S. pubescens ssp. *patula*	2*x*	1.48 ± 0.01 M-T
		S. pubescens ssp. *patula* 'Miss Kim'	2*x*	1.54 ± 0.01 H-S
Villosae	1.55 ± 0.02 B	*S. emodii*	2*x*	1.55 ± 0.01 G-S
		S. josikaea	2*x*	1.57 ± 0.01 E-R
		S. julianae	2*x*	1.59 ± 0.03 C-P
		S. sweginzowii	2*x*	1.55 ± 0.02 G-S
		S. tigerstedtii	2*x*	1.38 ± 0.01 T-U
		S. villosa	2*x*	1.56 ± 0.03 F-R
		S. villosa 'Aurea'	2*x*	1.62 ± 0.03 B-N
		S. wolfii	2*x*	1.57 ± 0.02 D-Q
		S. ×*prestoniae* 'Donald Wyman'	2*x*	1.50 ± 0.00 L-T
		S. ×*prestoniae* 'Miss Canada'	2*x*[u]	1.61 ± 0.03 B-O
		S. ×*prestoniae* 'Redwine'	2*x*	1.53 ± 0.02 I-T
		S. yunnanensis	2*x*	1.58 ± 0.01 C-Q
Ligustrina	1.49 ± 0.05 B	*S. pekinensis* China Snow®	2*x*	1.41 ± 0.02 R-U
		S. pekinensis Summer Charm®	2*x*	1.47 ± 0.03 N-U
		S. reticulata	2*x*[u]	1.59 ± 0.03 C-Q
Pinnatifoliae	1.52 ± 0.02 B*	*S. pinnatifolia* var. *alashanensis*	2*x*	1.52 ± 0.02 J-T

[z]Series designation based on phylogeny by Li et al. (2012).

[y]Series means based on average of taxa means; letters represent Tukey-Kramer test for unequal sample sizes ($\alpha = 0.05$); * = three samples of same accession were used to calculate mean.

[x]Taxa grouped within series; species and market name (cultivar or trademark) presented.

[w]Ploidy of each taxon; * = ploidy confirmed with root tip cytology.

[v]Means separated using Tukey's HSD ($\alpha = 0.05$); means followed by same letter are not significantly different; dash between letters indicate complete series of letters; minimum significant difference = 0.158.

[u]Ploidy confirmed with root tip cytology.

Table 3.4. Comparison of hybrid genome size from interploid and intraploid crosses in lilac (*Syringa*).

Parent ploidy[z]	Cross[y]	Accession no.[x]	Relative 2C genome size [mean ± SE (pg)][w]
3*x* x 2*x*	*S. vulgaris* 'President Grévy' x *S. vulgaris* 'Sensation'	H2013-150-01	5.65 ± 0.02 A
2*x* x 3*x*	*S. vulgaris* Blue Skies® x *S. vulgaris* 'President Grévy'	H2014-033-01	4.35 ± 0.03 B
		H2014-033-08	4.28 ± 0.05 BC
		H2014-033-04	4.25 ± 0.06 BC
		H2014-033-05	4.25 ± 0.03 BC
		H2014-033-12	4.07 ± 0.03 CD
		H2014-033-09	4.02 ± 0.13 CDE
		H2014-033-02	3.86 ± 0.05 DEF
		H2014-033-03	3.80 ± 0.06 DEF
		H2014-033-07	3.74 ± 0.05 EF
		H2014-033-10	3.74 ± 0.04 F
		H2014-033-06	3.58 ± 0.08 FG
		H2014-033-11	3.41 ± 0.03 GH
2*x* x 2*x*	*S. vulgaris* Tiny Dancer™ x *S. vulgaris* 'Sensation'	H2014-032-17	3.30 ± 0.05 H
		H2014-032-14	3.26 ± 0.01 H
		H2014-032-08	3.24 ± 0.03 H
	S. ×*hyacinthiflora* 'Old Glory' x *S. vulgaris* Tiny Dancer™	H2014-025-13	3.30 ± 0.03 H
	S. vulgaris Blue Skies® x *S. vulgaris* Tiny Dancer™	H2014-022-01	3.27 ± 0.04 H
		H2014-022-02	3.23 ± 0.06 H
		H2014-022-04	3.16 ± 0.04 H
	S. ×*hyacinthiflora* 'Old Glory' x *S. vulgaris* 'Angel White'	H2014-024-16	3.27 ± 0.08 H
		H2014-024-25	3.23 ± 0.04 H
		H2014-024-27	3.23 ± 0.02 H
		H2014-024-22	3.22 ± 0.04 H
		H2014-024-03	3.20 ± 0.03 H
	S. vulgaris 'Sensation' x *S. vulgaris* Tiny Dancer™	H2014-027-08	3.22 ± 0.03 H
		H2014-027-03	3.19 ± 0.06 H

[z]Ploidy of parent taxa including triploid by diploid (3*x* x 2*x*), diploid by triploid (2*x* x 3*x*) and diploid by diploid (2*x* x 2*x*) crosses.

[y]Crosses among cultivars in series *Syringa*; seed parent listed first and pollen parent listed second.

[x]Individual accessions in research population.

[w]Relative 2C holoploid genome sizes; means separated using Tukey's Honest Significant Difference test at ($\alpha = 0.05$; minimum significant difference = 0.286); means followed by the same letter are not significantly different.

Figures

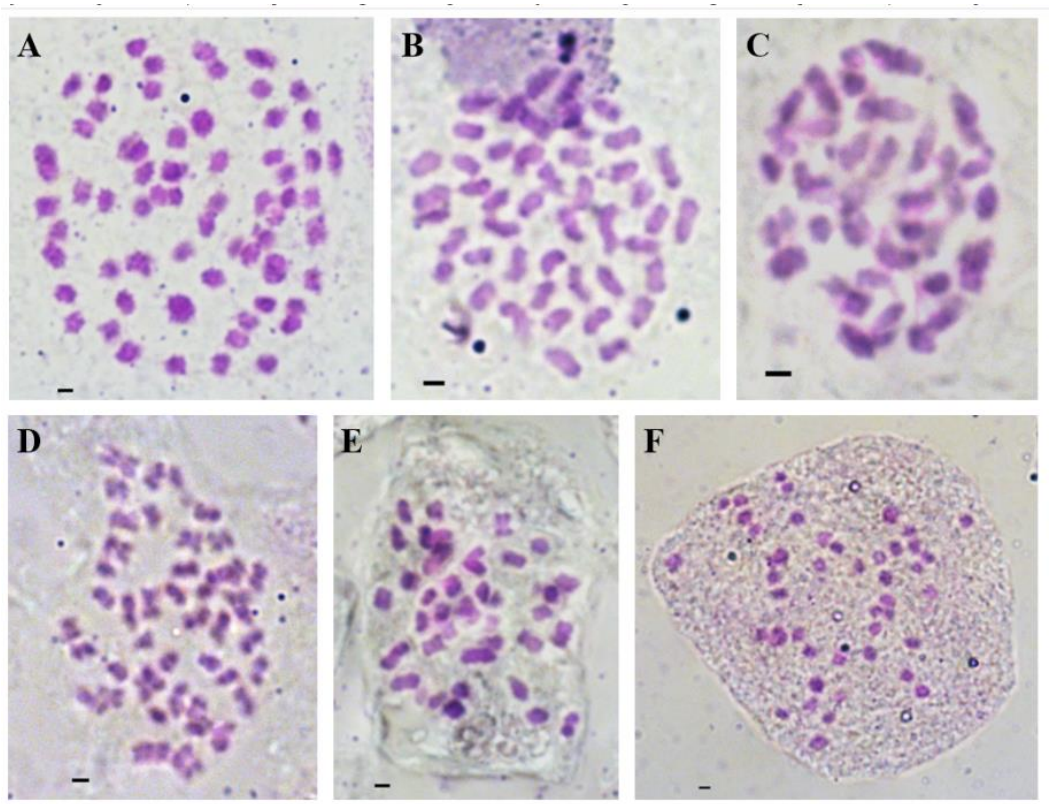

Fig. 3.1. Stained chromosomes in root tip cells of six accessions of lilac. Photomicrographs viewed at ×1000 with scale bar at 1 μm. (A) Triploid (2*n* = 3*x* = 69) *Syringa vulgaris* 'Aucubaefolia'. (B) Diploid (2*n* = 2*x* = 46) *Syringa* ×*hyacinthiflora* 'Maiden's Blush'. (C) Diploid (2*n* = 2*x* = 46) *Syringa* ×*hyacinthiflora* 'Old Glory'. (D) Diploid (2*n* = 2*x* = 46) Syringa ×prestoniae 'Miss Canada'. (E) Diploid (2*n* = 2*x* = 46) *Syringa reticulata*. (F) Diploid (2*n* = 2*x* = 46) *Syringa pubescens* Bloomerang® Purple.

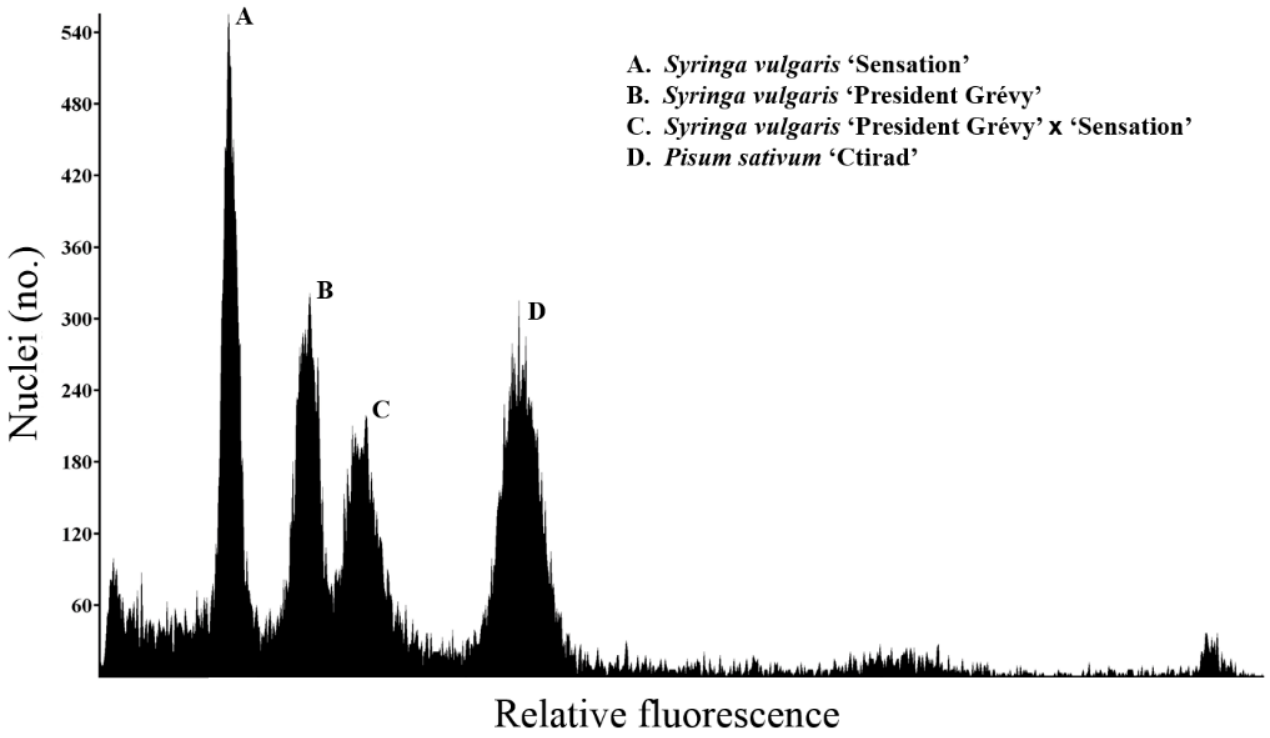

Fig. 3.2. Flow cytometry histogram of three taxa of *Syringa vulgaris* with an internal standard. (A) *Syringa vulgaris* 'Sensation' (2C relative genome size = 3.33 pg). (B) *Syringa vulgaris* 'President Grévy' (2C relative genome size = 4.85 pg). (C) Hybrid (H2013-150-01) *Syringa vulgaris* 'President Grévy' x *Syringa vulgaris* 'Sensation' (2C relative genome size = 5.65 pg). (D) Internal standard *Pisum sativum* 'Ctirad' (2C genome size = 8.76 pg).

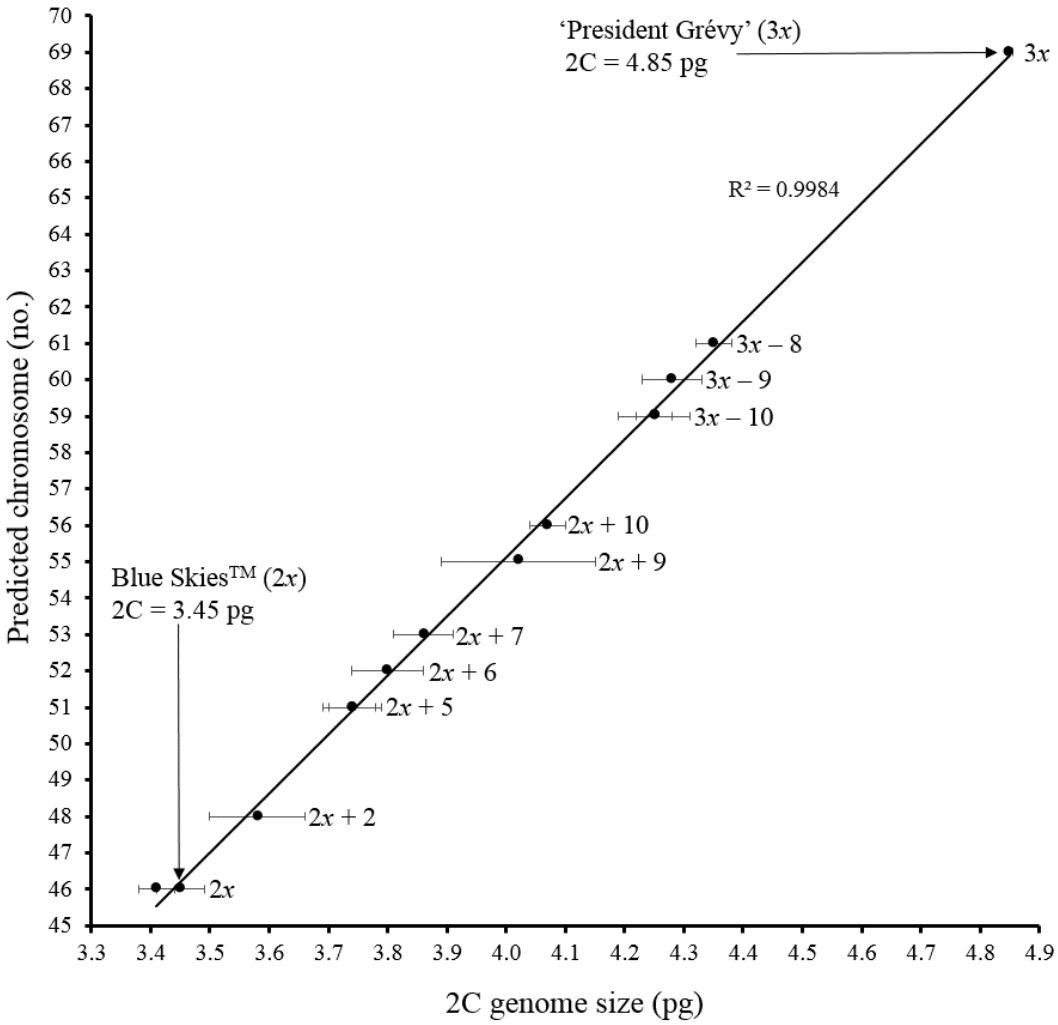

Fig. 3.3. Linear model of lilac 2*x* x 3*x* aneuploid progeny with predicted chromosome number based on theoretical chromosome size of 0.061 pg [(4.85 pg – 3.45 pg) / 23 chromosomes]. Parent taxa of aneuploid progeny: diploid female parent *Syringa vulgaris* Blue Skies™ (2*n* = 2*x* = 46) and triploid male parent *Syringa vulgaris* 'President Grévy' (2*n* = 3*x* = 69). Linear model follows the formula: y = 16.224x – 9.7743.

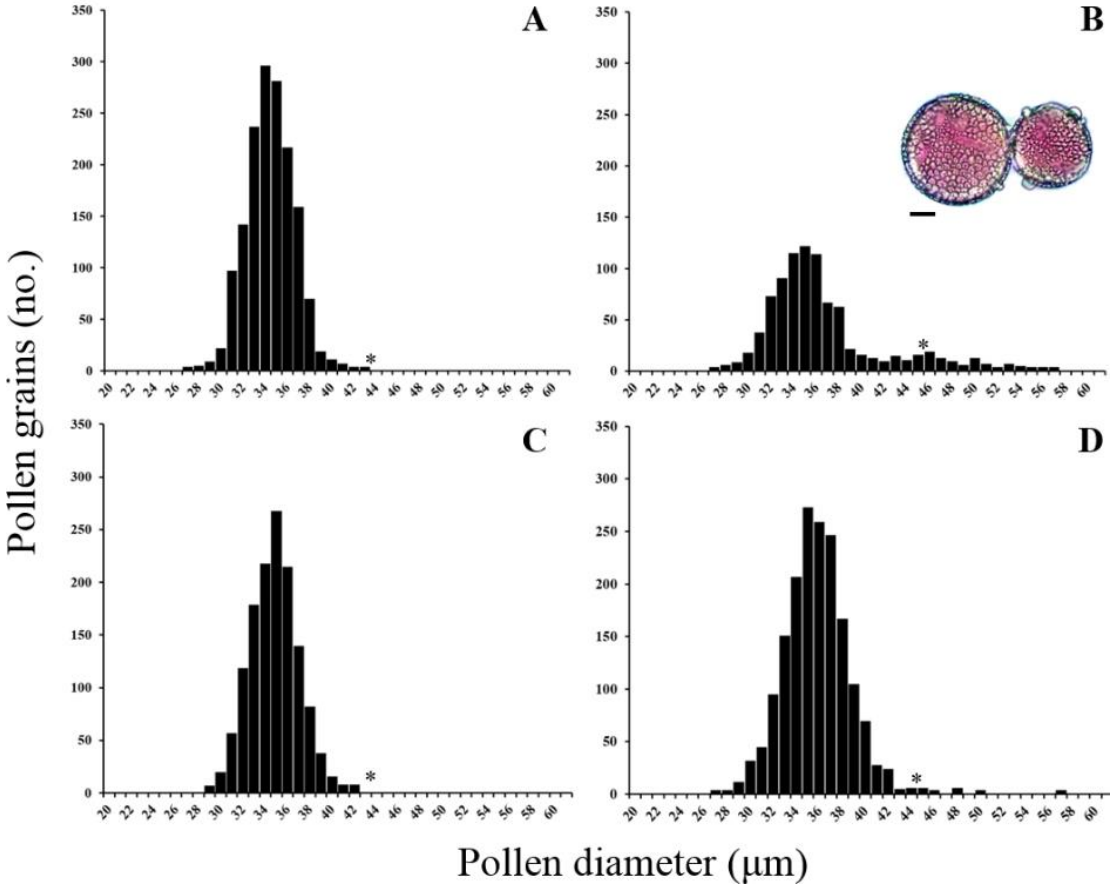

Fig. 3.4. Frequency distribution of viable pollen grain diameters of four cultivars of *Syringa vulgaris*. Regions to the right of asterisks were measured to be 26% larger than the mean and indicate unreduced gametes. (A) *Syringa vulgaris* 'Ludwig Spaeth'; 0% unreduced gametes. (B) *Syringa vulgaris* 'Sensation'; 8.5% unreduced gametes [Insert: Unreduced (left) and reduced (right) pollen grains stained with 2% acetocarmine and viewed at ×630 magnification; scale bar = 10 μm]. (C) *Syringa vulgaris* 'Miss Ellen Willmott'; 0% unreduced gametes. (D) *Syringa vulgaris* 'President Grévy'; 0.6% unreduced gametes.

CHAPTER 4: GREEN SEED GERMINATION AND REDUCED JUVENILITY IN COMMON LILAC.

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CHAPTER 4: GREEN SEED GERMINATION AND REDUCED JUVENILITY IN COMMON LILAC

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Additional index words:

Abstract. Common lilac, *Syringa vulgaris*, is an important flowering shrub for the US nursery industry. Cultivar improvement in common lilac has been a process of centuries, yet little research has focused on shortening generation time for lilac breeders. Juvenility is one of the major obstacles facing woody plant breeders. Several cultural techniques have been implemented to reduce the juvenility in woody plants, including direct-sowing of immature "green" seed. In a previous cross-compatibility study, observations of first-year hybrid seedlings were observed to undergo a quiescent phase of growth, producing few leaves and an extensive root system. This study investigated the effects of six germination and post-germination treatments on green seed (GS1, GS2, GS3, and GS4) and dry, dehisced seed (DS1, DS2, and DS3) on seed germination and subsequent plant growth in lilacs. These treatments varied based on seed maturity (green vs. dry), sowing date (weeks after pollination), capsule/seed color, seed treatment (direct-sown vs. cold-stratified), and post-germination treatments (greenhouse vs. post-germination chilling). Green seed treatment group GS4 reached an average of 80.00% germination. Green seed treatment groups GS3 and GS4 were not significantly different, and performed as well as cold-stratified dry seed (DS1). After the first full growing season, average

height of seedlings derived from direct-sown, mature seed with high moisture content (GS3 and GS4) was more than twice that of dry seed treatments (DS1, DS2, and DS3). These results indicate that green seed sowing has a significant effect on plant growth, and may provide a new tool for shortening juvenility and reducing breeding time in common lilac.

Introduction

Common lilac, *Syringa vulgaris*, is a clonally propagated, woody shrub that has been the subject of intense breeding for centuries due to its fragrant, colorful spring blooms (Fiala and Vrugtman, 2008). The majority of species originate in Asia with only *S. vulgaris* and *S. josikaea* originating in Europe (Fiala, 1988). The center of diversity of common lilac lies in the Balkans while native populations can be found throughout southeastern Europe (Fiala, 1988). The impact of lilacs to the US nursery industry cannot be understated; nearly $20 million in revenues were generated from over 1.8 million sales in 2014 (USDA, 2014).

A major limiting factor in woody plant breeding is the length of time between successive generations. Juvenility in woody plants is a natural process that prevents flowering in seedlings. Woody plants in nature establish years of vegetative growth under competition before diverting resources to fruit and seed production (Van Nocker and Gardiner, 2014). In the artificial environment of a breeding program, breeders have several cultural techniques for circumventing this natural defense mechanism. One method is to provide optimal growing conditions to promote vigorous, upright vegetative growth. Apples breeders growing seedlings under

optimal conditions have reduced juvenility to ten months compared to five years for field-grown seedlings (Aldwinckle, 1975). Applying extreme conditions such as water stress and mineral deficiencies has been used to induce flowering, but can also delay the transition to maturity by limiting vigorous growth (Aldwinckle, 1975; Zimmerman, 1972). Another method is the use of plant growth regulators (PGRs), though this approach has proven highly variable and has not been widely adopted for reducing juvenility (Zimmerman et al., 1985). However, for large trees with extreme juvenility periods, combinations of PGR applications, root restriction and girdling have proven effective for reducing juvenility (Philipson, 1996; Snowball et al., 1994). Once seedlings reach maturity, forcing is a cultural method that can be used to trigger vegetative and floral development in lilacs using high temperatures from 37 °C in November to 16 °C in March (Jędrzejuk et al., 2016b). High temperatures required for forcing often degrade pollen grains and ovules, making this technique problematic for breeding (Jędrzejuk et al., 2016b). However, current research is proving that low temperature forcing (near 15 °C in November) reduces oxidative stress in lilac flowers, which may prove useful for lilac breeders (Jędrzejuk et al., 2016a).

Though cultural techniques exist for reducing generation time in woody plants, most focus on treatments to germinated seedlings. Efforts to overcome lengthy periods of seed dormancy have also proven effective, including “green” seed germination, embryo culture, bioactive gibberellic acid treatments, and nitric oxide treatments (Bethke et al., 2007; Bridgen, 1994; Shen et al., 2011; West et al., 2014; Van Nocker and Gardiner 2014). Late fall green seed collection has proven an effective method to promote germination of seeds in several woody plant taxa,

including *Tilia americana* (Dirr and Heuser, 2006) and *Syringa reticulata* (West et al., 2014). Seed development and depth of seed dormancy vary in lilacs due partly to genetic variation and partly to environmental conditions such as temperature post-pollination (Junttila, 1973). In tree lilac (*S. reticulata*), seeds were determined to be fully mature and capable of germinating as the green capsule color began to fade (West et al., 2014). Germination was optimized by West et al. (2014) by collecting capsules at one week intervals just as the green color began to fade in early Fall. Germination diminished precipitously as moisture content was lost (West et al., 2014).

In a previous study on cross compatibility among lilac cultivars (Lattier and Contreras, 2017), germinated seedlings from cold-stratified seed were observed to have a quiescent phase their first year where vegetative growth was limited while the seedling developed a large root system (Fig. 4.1A). Seedlings produced a large flush of vegetative growth in their second year (J. Lattier – personal observation). In 2014, a preliminary trial was conducted on immature seed germination. Pre-dehisced green, yellow-green, and yellow capsules were collected in summer from a random crosses of a cross-compatibility study in lilac (Lattier and Contreras, 2017). Seeds were direct-sown into 1.3-L containers (Fig. 4.1B) as well as direct-sown on Petri dishes with moistened filter paper (Fig. 4.1C). Only seeds excised from yellow-green and yellow capsules germinated in both Petri dishes and containers. Direct-sown seeds grew to their quiescent phase in a glasshouse and were then moved to an unheated polyhouse for winter dormancy. In spring, these seedlings produced a large flush of vegetative growth and quickly achieved the same size as stratified seedlings from the

previous year (J. Lattier, personal observation). These preliminary results provided a proof of concept that germinating immature seed may be a means of reducing generation time for lilac breeders.

The purpose of this study was to 1) determine the optimum germination treatment to break the quiescent phase before the first year of growth, and 2) determine if germination treatments improved growth and reduced juvenility in common lilac.

Methods and Materials

Plant Materials. In the spring of 2015, two elite lilac cultivars were selected based on their reported cross compatibility in a previous study (Lattier and Contreras, 2017). *S. vulgaris* 'Ludwig Spaeth' (10-0042) and *S. vulgaris* 'Angel White' (10-0043) were acquired from Blue Heron Farms in Corvallis, Oregon. In early spring, prior to anthesis, parent cultivars were placed in a glasshouse, kept free of pollinators, and grown under day/night temperatures of 25/20 °C and a 16-h photoperiod. The pollen parent, *S. vulgaris* 'Angel White' was moved into the heated glasshouse approximately two weeks before the seed parent, *S. vulgaris* 'Ludwig Spaeth', to hasten flower development.

Germination Experiment. As flowers of *S. vulgaris* 'Angel White' reached anthesis, fresh pollen was collected and stored in small Petri dishes over desiccant (Drierite™; W.A. Hammond Drierite Co. Ltd., Xenia, OH) in a refrigerator at 4 °C. Two to four anthers from each flower were collected; no petal tissue was stored with the pollen. Prior to pollination, open flowers on *S. vulgaris* 'Ludwig Spaeth' were

removed on all inflorescences. Individual flowers on multiple inflorescences were emasculated prior to anthesis. Each flower was pollinated using a small paintbrush two to three times post-emasculation over consecutive days. All inflorescences were marked with jewelry tags and labelled with the cross combination, date, and number of flowers pollinated. Over the course of a single day, over 600 flowers were pollinated on 13 inflorescences; all other inflorescences were removed.

Developing capsules were randomly removed at intervals of five weeks after pollination (WAP) until 20 WAP. At 20 WAP, half the capsules on *S. vulgaris* 'Ludwig Spaeth' were mature and dehiscent. Each pre-dehisced, immature capsule was collected (Fig. 4.2A) and immediately surface-sterilized by soaking in 70% ethanol for one minute (Fig. 4.2B). Capsules were placed in a sterile sieve and thoroughly rinsed with sterile, distilled water (Fig. 4.2C). Capsules were temporarily stored (less than 2 h) in vials of distilled water until seed extraction. Seeds were carefully excised on sterile paper plates using a scalpel and forceps (Fig. 4.2D). Excised seeds were temporarily stored (less than 2 h) in distilled water until sown (Fig. 4.2E). Dehisced, mature capsules were collected and dry seed was direct-sown or allowed to completely dry and then placed into cold-stratification (Fig. 4.2F). WAP was recorded and morphological data such as seed color (which corresponded with capsule color), and phase of seed development (turgid, green seed or dry, dehisced seed) were noted (Table 4.1). For each treatment, 60 seeds were divided into four lots of 15 seeds and sown into mum pots in a growing medium (Metro-Mix; Sun Gro Horticulture, Agawam, MA). Seeds were planted at ~1/2 inch under the surface of the medium and pots were completely randomized. Seeds were germinated

in a glasshouse under the conditions described above. For each WAP interval, the process of capsule collection, seed excision, and sowing took place over a single day.

At 20 WAP, half of the capsules on *S. vulgaris* 'Ludwig Spaeth' were dehisced and half were still immature and pre-dehisced. Two experimental groups were direct-sown at 20 WAP from these two types of capsules and the remaining dehisced capsules were bulk-collected (Table 4.1). This concluded the sequential removal of capsules from the seed parent leaving four immature, green seed treatment groups and one mature, dry seed group: GS1, GS2, GS3, GS4, DS1 (Table 4.1). A third experimental group (DS2) was created from the 20 WAP bulk seed for comparing germination of direct-sown seed with germination of cold-stratified seed (Table 4.1). Four seed lots were sown in mum pots as described above and placed in a cooler at 4 °C for ten weeks before being returned to the glasshouse. Plants were germinated and grown under glasshouse conditions for the remainder of the winter. Germination rates for each treatment group were recorded after a minimum of one month (Fig. 4.3), meeting the prescribed 21-day requirement for lilac germination tests (Isely and Everson, 1965; ISTA 1966).

Post-Germination Experiment. Treatments groups that were direct-sown and produced viable seedlings (GS2, GS3, GS4, DS1) were transplanted into quart containers and grown in a glasshouse through late summer and early fall. They were then placed in an unheated polyhouse and allowed to go dormant for the winter. The cold-stratified treatment group (DS2) was germinated under glasshouse conditions, potted into quart containers, and grown under glasshouse conditions throughout the winter. A fourth experimental group (DS3) was created from the 20 WAP bulk seed

for determining the effect of post-germination chilling on seedling growth and development (Table 4.1). This treatment group was cold-stratified, germinated in a glasshouse, and given a 60-day post-germination chilling period at 4 °C. After post-germination chilling, these plants were grown in a glasshouse for the remainder of winter. Observations on winter defoliation and mortality were recorded prior to the first growing season (Fig. 4.4). In spring, all plants were moved to an outdoor lath structure for their first full growing season. Pots derived from six treatment groups (GS2, GS3, GS4, DS1, DS2, DS3) were completely randomized and grown throughout the 2016 growing season in Corvallis, OR. At the end of the growing season, when plants began to go dormant in fall, plant height was measured in millimeters from the soil surface to top of the tallest branch on each plant (Fig. 4.5).

Statistical analysis. For the germination experiment, observations were made on six treatment groups: GS1, GS2, GS3, GS4, DS1, and DS2. Treatment group GS1 did not produce any germinated seedlings and was excluded from analysis of variance (ANOVA) (SAS Studio; Cary, NC). Percent germination means were normally distributed and passed Levene's test for homogeneity of variance (F = 1.57; P = 0.23). Means were separated using Tukey's Honest Significant Difference (HSD) test (α = 0.05) with a minimum significant difference of 29.66% germination (Fig. 4.6). Error terms are reported as standard error of means (SEM).

For the post-germination experiment, observations were made on six treatment groups: GS2, GS3, GS4, DS1, DS2, and DS3. Due to low germination rates in the first experiment, treatment group GS2 had a small sample size (N = 3) and was excluded from the ANOVA. Mean heights (mm) were normally distributed, yet

failed Levene's test for homogeneity of variance (F = 9.34; P < 0.0001). After a natural log transformation, treatment means [ln(mm)] passed Levene's test (F = 0.73; P = 0.5702). Least squares means are reported from log-transformed data, back-transformed [$e^{ln(mm)}$] to the original scale (Fig. 4.7). Least squares means were separated using Tukey-Kramer test (α = 0.05) for unequal sample sizes. Error terms were reported from 95% confidence intervals back-transformed to the original scale (Fig. 4.7).

Results and Discussion

Germination experiment. Ease of seed extraction varied across treatment groups. Early seed extraction in treatment groups GS1 and GS2 required delicate removal of capsule from the seed; great care was taken not to damage the immature, green seed (Fig. 4.2D). Seeds were easily damaged if touched with forceps and were tightly held in the locules. Seed extraction became easier in treatments with longer WAP periods, with GS3 and GS4 requiring less invasive cutting of the capsule. For GS3 and GS4, seeds were removed by lightly scoring the capsule sutures with a scalpel, piercing the proximal end the capsule with the scalpel tip, and twisting the scalpel until the capsule opened. Seeds were less tightly held in the locules and would often fall out after opening the capsules. The easiest seed extraction was from dry, dehisced capsules of treatment groups DS1 and DS2. Dried seeds could be handled without damage (Fig. 4.2F).

Treatments produced significant differences in percent germination (P < 0.001) in the first experiment. Direct-sown, green seed treatment groups GS1

through GS4 varied from 0% to 80% germination. Treatment group GS1 failed to produce any germinated seedlings while all other treatments produced viable seedlings (Fig. 4.3). Of the treatments groups that produced viable seedlings, GS2 had the lowest percent germination at 6.67% (Fig. 4.6). Treatment groups GS3 and GS4 were not significantly different. Treatment group GS3 had an average of 58.33 % germination. Treatment group GS4 had the largest observed percent germination at 80.00% which was significantly higher than treatment group DS1 (41.67%) collected at the same WAP (Fig. 4.6). This agrees with previous reports in tree lilacs that mature, yellow capsules and seed with high moisture content collected late in WAP period produce high germination rates compared with dry seed (West et al., 2014). Treatment groups GS3 and GS4 were not significantly different than the cold-stratified treatment group GS2 (Fig. 4.6). Therefore, GS3 and GS4 performed equally well compared to the traditional method of lilac seed germination, which recommends a minimum of two months of cold-stratification (Dirr and Heuser, 2006). Early germination of treatment groups GS2, GS3, GS4, and DS1 allowed them to grow into the quiescent state prior to their first winter. Seed from treatment group DS2 was allowed to completely dry and placed in cold-stratification during winter.

Post-germination experiment. Winter observations of the direct-sown treatment groups GS2, GS3, GS4, and DS1 revealed that all plants retained their foliage during their first winter dormancy except for plants from DS1 (Fig. 4.4). Spring vegetative budbreak was observed and plant survival was recorded. All plants from treatment groups GS2, GS3, and GS4 survived winter dormancy and only one

plant from the defoliated DS1 group died. Mortality was high in the DS3 treatment group, which was given a 60-day, post-germination chilling after cold stratification and germination. Seven out of the 30 treated plants died, yielding a mortality rate of 23.33%. Plant height was recorded in the fall after the first full growing season.

Seedlings derived from the direct-sown, green seed treatment groups (GS2, GS3, and GS4) were observed to produce larger, more heavily branched plants than the dry seed treatment groups (DS1, DS2, and DS3) (Fig. 4.5). The largest plants were observed in treatment group GS2; however, low germination rates from the first experiment yielded only three plants to evaluate. Therefore, GS2 was excluded from statistical comparisons. Germination and post-germination treatment groups produced significant differences in plant height ($P < 0.001$) in the second experiment. Treatment group GS3 and GS4 produced significantly taller plants than DS1, DS2, and DS3 after one complete growing season (Fig. 4.7). The average height of GS3 was at 130.75 mm within a 95% confidence interval of 118.10 to 144.75 mm. The average height of GS4 was 148.97 mm within a 95% confidence interval of 136.69 to 162.34 mm (Fig. 4.7). Treatment groups DS1, DS2, and DS3 failed to achieve half of the size of GS3 and GS4 with the largest average height in DS3 at 42.41 mm within a 95% confidence interval of 37.41 to 48.08 mm (Fig. 4.7).

The germination experiment in 2015 was successful at revealing differences in germination among treatments. In summer, as capsules and seeds fade from green to yellow at 15 to 20 WAP, mature seed with high moisture content can be extracted and a high germination rate equal to the traditional method of dry, cold-stratified seed can be achieved. The benefit of avoiding months of drying and cold-stratifying seed

allowed the seedlings to grow into the first-year quiescent phase, producing several sets of leaves and an extensive root system prior to going dormant for winter. The post-germination experiment in 2016 revealed differences in plant height from germination and post-germination treatments. The average height of seedlings derived from direct-sown, mature seed with high moisture content was observed to be more than twice that of the dehisced seed treatments in the first growing season. In addition to an increase in plant height, we observed that these treatments produced larger, more heavily branched plants. The results of this experiment show significant gains in growth by direct-sowing summer seed from hybrid lilac crosses. Over the next several years, data will be collected on flowering time of each hybrid progeny and average juvenility will be compared across germination and post-germination treatment groups. If plant growth continues to follow the trend observed after the first full growing season, green seed sowing might be a valuable method for shortening juvenility. Currently, one generation from seed to flowering plant requires three to five years. This technique, combined with other cultural practices, and may significantly increase the number of generations per cycle for breeders of common lilac.

Literature Cited

Bethke, P.C., I.G. Libourel and R.L. Jones. 2007. Nitric oxide in seed dormancy and germination. In: Annual Plant Reviews. Seed Development, Dormancy and Germination. Vol. 27:153-175.

Bridgen, M.P. 1994. A review of plant embryo culture. HortScience 29:1243-1246.

Dirr, M.A. and C.W. Heuser. 2006. The reference manual of woody landscape propagation: from seed to tissue culture. 2nd ed. Timber Press, Portland, OR.

Fiala, J.L. and F. Vrugtman. 2008. Lilac: a gardener's encyclopedia. Timber Press, Inc. Portland, OR.

Fiala, J.L. Lilacs. 1988. The genus *Syringa*. Timber Press, Inc. Portland, OR.

Isely, D. and L.E. Everson. eds. 1965. Rules for testing seeds. Proc. Assn. Offic. Seed Analysts 54:1–112.

ISTA [International Seed Testing Association]. 1966. International rules for seed testing. Proc. Intl. Seed Testing Assn. 31:1–152.

Jędrzejuk, A., A. Łukaszewska, J. Rabiza-Świder, and E. Skutnik. 2016a. Low temperature forcing reduces oxidative stress in lilac flowers. Hort. Environ. Biotechnol. 57: 625-632.

Jędrzejuk, A., J. Rabiza-Świder, E. Skutnik, and M. Serek. 2016b. Flowering conditions affect flower longevity in *Syringa vulgaris* and cause changes in protein content, protease activity and expression of KDEL-CysEP gene. Acta Physiol. Plant. 38:45 DOI: 10.1007/s11738-015-2044-z.

Junttila, O. 1973. Seed and embryo germination in *Syringa vulgaris* and *S. reflexa* as affected by temperature during seed development. Physiol. Plant. 29:264-268.

Lattier, J.D. and R.N. Contreras. 2017. Intraspecific, interspecific, and interseries cross-compatibility in lilac. J. Amer. Soc. Hort. Sci. 142: 279-288.

Philipson, J.J. 1996. Effects of girdling and gibberellin $A_{4/7}$ on flowering of European and Japanese larch grafts in an outdoor clone bank. Canadian J. For. Res. 26:355-359.

U.S. Department of Agriculture (USDA). 2016. Census of horticultural specialties for 2014. 15 July 2016. https://www.agcensus.usda.gov/Publications/2012/Online_Resources/Census_of_Horticulture_Specialties/.

Van Nocker, S. and S.E. Gardiner. 2014. Breeding better cultivars, faster: applications of new technologies for the rapid deployment of superior horticultural tree crops. Hort. Res. 1:14022. doi:10.1038/hortres.2014.22.

West, T.P., S.L. DeMarais, and C.W. Lee. 2014. Germination of nonstratified Japanese tree lilac as influenced by seed capsule maturity and moisture content. HortScience 24: 177-180.

Shen, X., F.G. Gmitter, Jr. and J.W. Grosser. Immature embryo rescue and culture. Methods Mol. Biol. 710:75-92.

Snowball, A.M., I.J. Warrington, E.A. Halligan and M.G. Mullins. 1994. Phase change in citrus: the effects of main stem node number, branch habits and paclobutrazol application on flowering in citrus seedlings. J. Hort. Sci. Biotechnol. 69:149-160.

Zimmerman, R.H. 1972. Juvenility and flowering in woody plants: a review. HortScience 7:447-455.

Zimmerman, R.H., W.P. Hackett and R.P. Pharis. 1985. Hormonal aspect of phase change and precocious flowering. Encycl. Plant Physiol. 11:79-115.

Tables

Table 4.1. Description of treatment groups for germination and post-germination experiments.

Treatment[z]	WAP[y]	Seed Color[x]	Seed development[w]	Germination treatment[v]
GS1	5	Green	Green Seed	Direct Sow
GS2	10	Yellow-Green	Green Seed	Direct Sow
GS3	15	Green-Yellow	Green Seed	Direct Sow
GS4	20	Yellow	Green Seed	Direct Sow
DS1	20	Brown	Dry Seed	Direct Sow
DS2	20	Brown	Dry Seed	Cold Stratified
DS3	20	Brown	Dry Seed	Cold Stratified + Post-Germination Chilling

[z]Abbreviated name designating treatment group.

[y]Number of weeks after pollination (WAP) capsules were removed from seed parent, *Syringa vulgaris* 'Ludwig Spaeth'.

[w]Phase of seed development: immature "green" seed or physiologically dormant dry seed.

[v]Germination treatments including direct sowing, ten-week cold stratification, and ten-week cold stratification plus 60 days post-germination chilling.

Figures

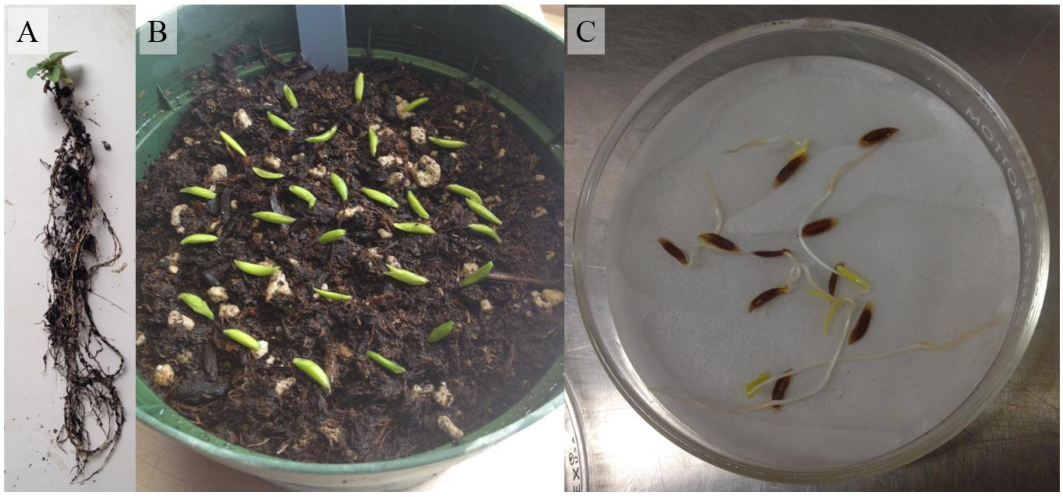

Fig. 4.1. Preliminary observations on lilac seeds and seedlings. (A) Quiescent seedling derived from cold-stratified seed during the first year of growth. (B) Immature extracted seed in mum pots from a 2014 preliminary trial of direct-sown summer lilac seed (C) Immature seed germinating on dampened filter paper in a Petri dish from a 2014 preliminary trial of direct sown summer lilac seed.

Fig. 4.2. Seed collection and extraction process for green seed (GS) treatment groups GS1, GS2, GS3, GS4 (A-E) and dry seed (DS) treatment groups DS1, DS2, and DS3 (F). (A) Collection of immature, pre-dehisced capsules. (B) Surface sterilization in 70% ethanol for one minute. (C) Rinse with sterile, distilled water. (D) Excision of immature green seed. (E) Storage of green seed in sterile, distilled water prior to sowing. (F) Collection of mature, dehisced capsules and dry seed.

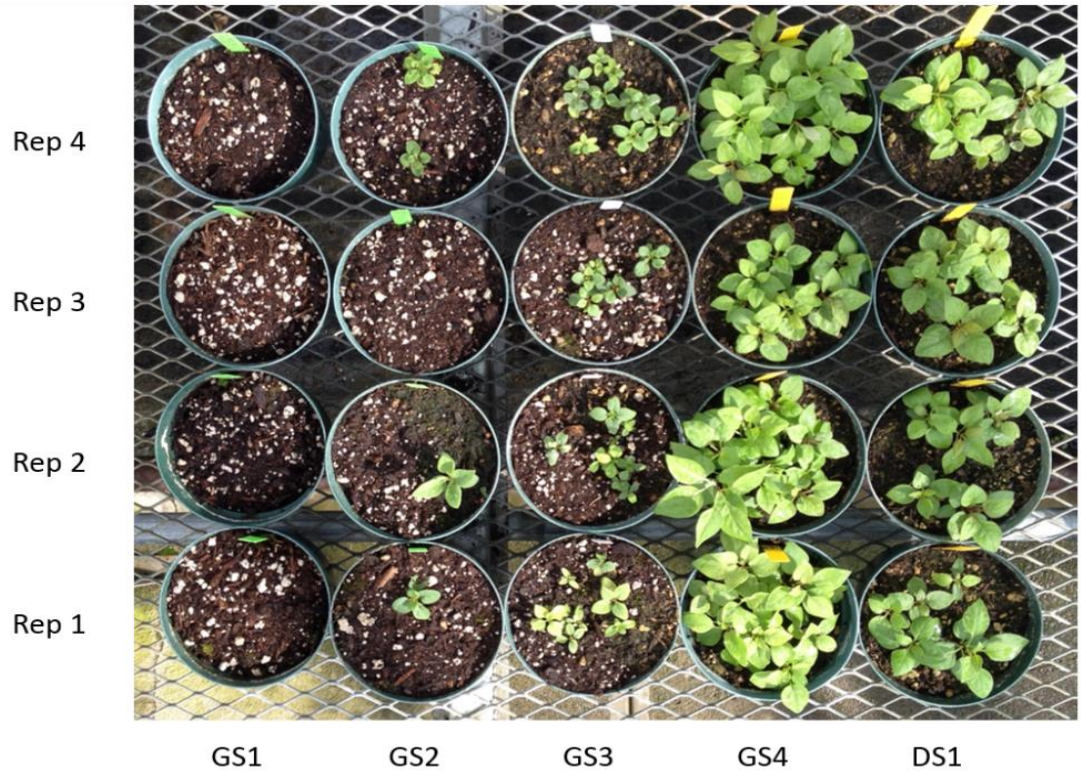

Fig. 4.3. Germination of direct-sown, green seed (GS) and dry seed (DS) treatment groups GS1, GS2, GS3, GS4 and DS, one month after sowing. Treatments consist of 60 seeds divided into lots of 15 seeds sown in mum pots.

Fig. 4.4. Winter comparison of foliated lilac seedlings from GS4 with defoliated seedlings from DS1 treatment group. (A) Dormant seedlings from treatment group GS4. (B) Dormant seedlings from treatment group DS1.

Fig. 4.5. Lilac seedlings from treatment groups GS2, GS3, GS4, DS1, DS2, and DS3. Seedlings displayed from each seed treatment group representing the average plant height after the first full growing season in quart containers. Scale bar at 500 mm. From left to right: GS2 (H2015-800-01), GS3 (H2015-800-11), GS4 (H2015-800-173), DS1 (H2015-800-97), DS2 (H2015-800-151), DS3 (H2015-800-135).

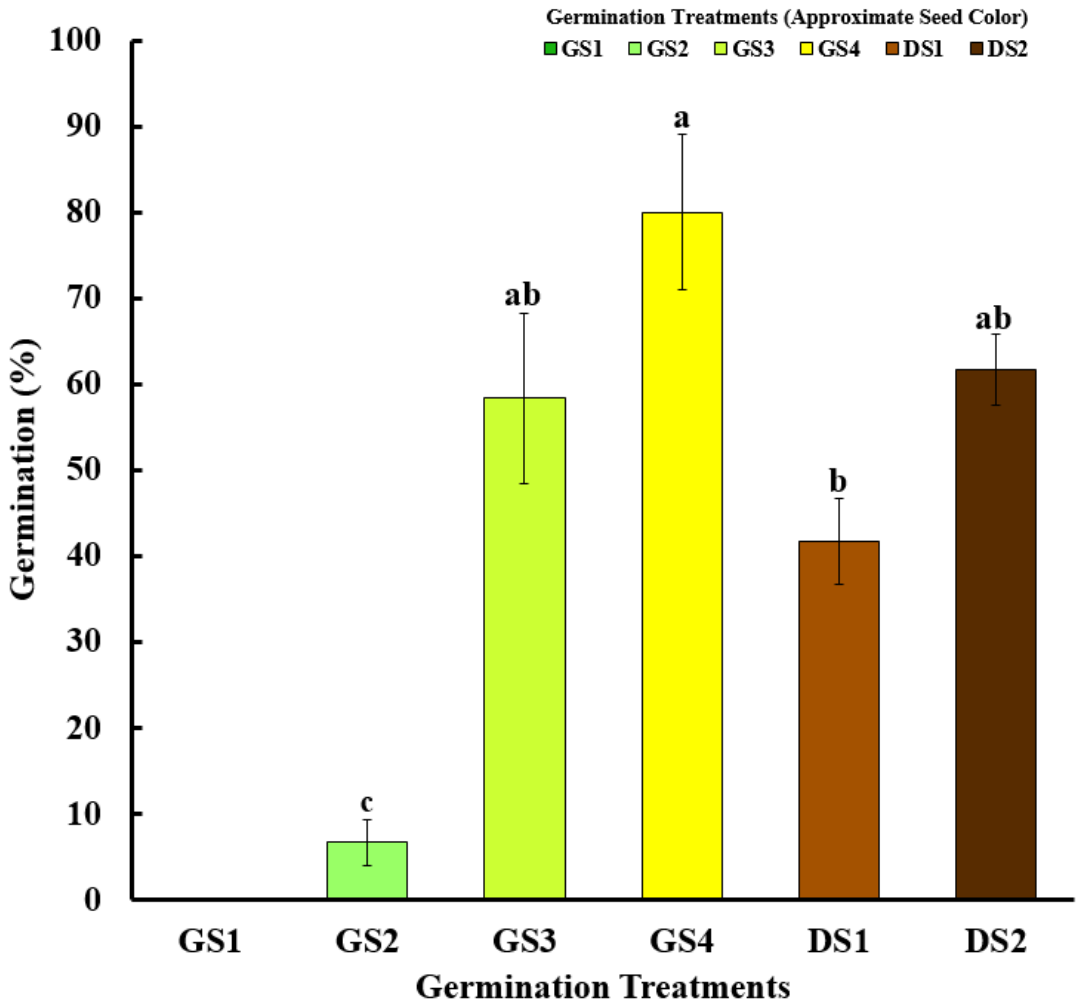

Fig. 4.6. Percent germination for six germination treatments. Means and standard errors are reported in percent germination. Letters above bars represent a Tukey's Honest Significant Difference (HSD) test ($\alpha = 0.05$) with a minimum significant difference of 29.66% germination. Bar colors represent an approximation of the original seed/capsule color from germination treatments.

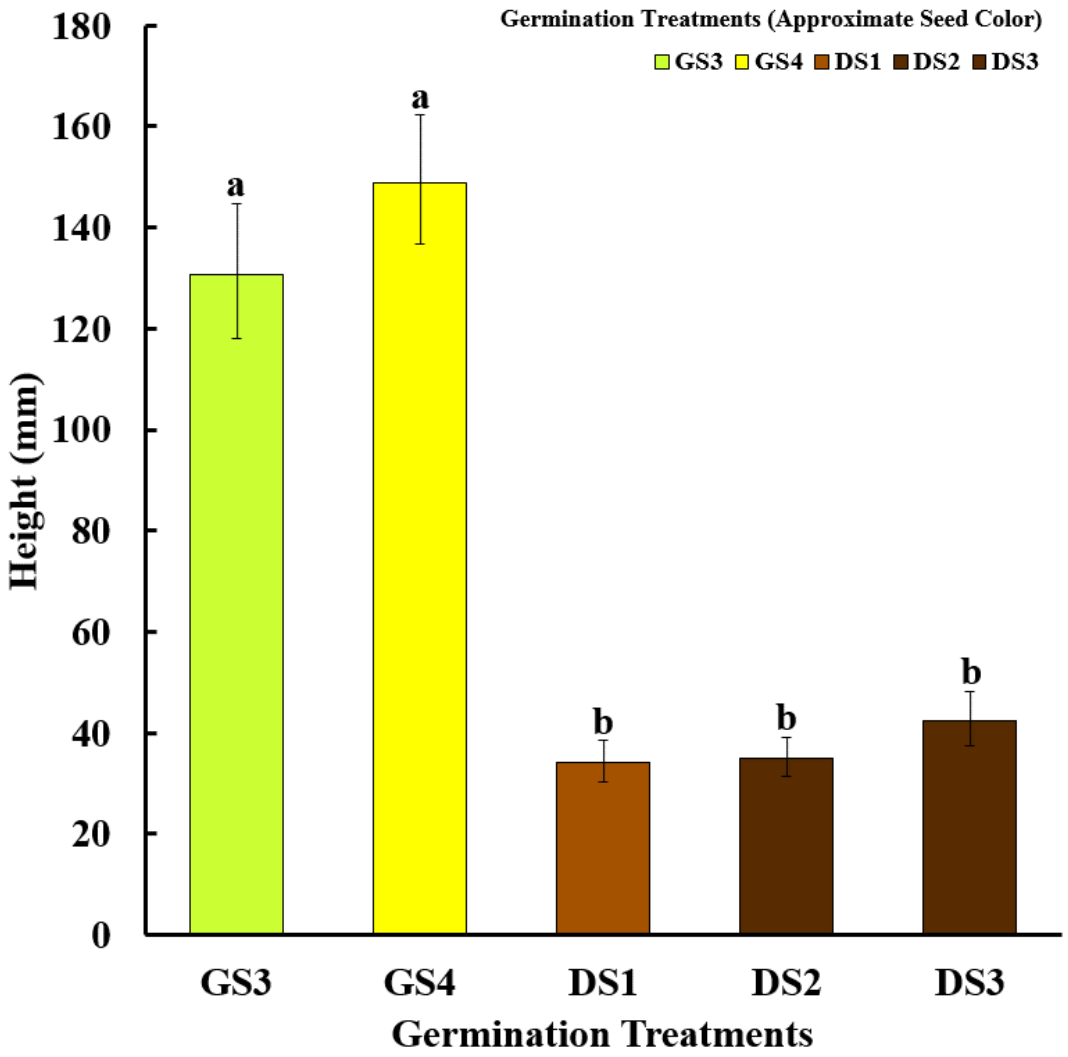

Fig. 4.7. Seedling height after one full growing season. Least squares means reported from log-transformed data, back-transformed to the original scale (mm). Error bar represent 95% confidence interval back-transformed to the original scale (mm). Letters above bars represent a Tukey-Kramer means separation for unequal sample sizes on log-transformed height data. Bar colors represent an approximation of the original seed/capsule color from germination treatments.

CHAPTER 5: PRELIMINARY SNP-BASED GENETIC LINKAGE MAPS OF *Syringa meyeri* ‘Palabin’ AND *S. pubescens* ‘Penda’ Bloomerang®.

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Additional index words: remontant, genotyping-by-sequencing, single nucleotide polymorphism, HapMaps, linkage groups

Abstract. The Oleaceae represents a diverse family of trees, shrubs, and lianas with nearly 600 species and 25 genera, including many economically important taxa. Within Oleaceae, lilacs are an important group of fragrant, woody ornamental shrubs that are a staple for the U.S. nursery industry. Lilacs exist primarily as diploids ($2n = 2x = 46$). Dwarf lilacs in series *Pubescentes* exhibit a range of important ornamental traits, such as summer reblooming (remontant) and disesease resistance. Many of these traits take years to develop in hybrid progeny of *Syringa* and breeders would benefit from genetic markers to identify beneficial traits at the seedling stage. The goal of this study was to develop a bi-parental mapping population from the cross of a remontant and non-remontant parent that exhibited variability in their resistance to pathogens such as bacterial blight. A mapping population resulting from the cross, *S. meyeri* 'Palibin' x *S. pubescens* 'Penda' Bloomerang® was mined for segregating single nucleotide polymorphisms (SNPs) using genotyping-by-sequencing (GBS). The double pseudo-testcross method was used to construct preliminary linkage maps for each parent. From an initial 20,729 SNP markers, 31% were used in construction of the parental linkage maps. Markers segregating 1 : 1 were used for mapping in

each parent, and cosegregating 1 : 2 : 1 makers were used to group equivalent linkage groups in each parental map. A maximum likelihood approach was used to generate putative linkage groups. Groups were selected that contained large numbers of markers at a LOD score of 10. For the female map 22 linkage groups (P1 – P22) were selected, and for the male map, 25 linkage groups were selected (B1 – B25). Both of these maps contain linkage groups near the true number of haploid chromsomes for dwarf lilac, $n = 23$. The preliminary parental maps were large with 'Palabin' at 11,492 cM and Bloomerang® at 8,717 cM. Maps were densly populated with SNP markers, at an average marker distance of 3.4 cM and 3.2 cM, respectively. Few genetic linkage maps exist for economically important ornamental crops, which are often complicated by polyploidy and large genomes. We report the first preliminary genetic linkage maps for dwarf lilacs. Work is currently underway to deep-sequence Bloomerang® to resolve problems with the preliminary map as well as develop novel SSR markers. Work is also underway to phenotype the mapping population for a range of ornamental traits for future use in marker-trait association mapping.

Introduction

The Oleaceae represents a diverse a family of trees, shrubs, and woody climbers with nearly 600 species and 25 genera (Wallander and Albert, 2000). Many economically important species belong to this family including fruit and oil crops, such as olive (*Olea*), and ornamental trees and shrubs including jasmine (*Jasminum*), forsythia (*Forsythia*), privet (*Ligustrum*), and lilac (*Syringa*). Lilacs are primarily

diploids with basic chromosome numbers reported at x = 22, 23, or 24 (Darlington and Wylie, 1956). Most taxa investigated proved to be the x = 23 cytotype, which likely arose from allopolyploidy between ancestral Oleaceae taxa of two cytotypes, x =11 and x = 12 (Stebbins, 1940; Taylor, 1945). Few studies exist on chromosome numbers in modern lilac cultivars. However, a recent study confirmed the popular, remontant (summer re-blooming) taxon, *S. pubescens* Bloomerang® to be a diploid, at $2n = 2x = 46$ and a genome size of 1.46 ± 0.03 pg (Lattier and Contreras, 2017).

Economically important floral traits, such as double flowers and remontant flowering, take years to develop in hybrid progeny of *Syringa*. Breeders currently dedicate resources including time and field space to grow large populations before evaluating floral traits. Disease susceptibility and resistance can also take years to evaluate in breeding populations of woody plants. Resistance to major pathogens such as powdery mildew, bacterial blight (*Pseudomonas syringae*), and foliar blight (*Alternaria*) has been shown to vary widely in species and cultivars of lilac, with some taxa (e.g. *S. meyeri* 'Palibin') showing moderate to complete resistance to all three pathogens (Mmbaga and Sheng, 1997; Mmbaga et al., 2005; Mmbaga et al., 2011). Having candidate markers for important flowering and disease resistance traits in lilac would greatly benefit breeders.

Previous research has yielded few markers for *Syringa*. Juntheikki-Palovaara et al. (2013) discovered nine polymorphic microsatellite markers for *S. vulgaris* from ISSR primers. De La Rosa et al. (2002) discovered seven microsatellite markers for olive (*Olea europaea* L.) and found that four amplified in *S. vulgaris* with two being polymorphic. Rzepka-Plevneš et al. (2006) tested 30 ISSR primers on seven different

species of *Syringa* and found 13 primers produced bands in all species with 109 ISSR fragments being polymorphic and 57 ISSR fragments being species specific. Lendvay et al. (2013) tested microsatellite primers from closely related genera (*Olea* and *Ligustrum*) on *S. josikaea* and found two were easily amplified and variable. They also developed novel primers for *S. josikaea* and found five microsatellites were easy to amplify and score (Lendvay et al., 2013). Kochieva et al. (2004) conducted a RAPD analysis of six species, 15 cultivars, and one interspecific hybrid in *Syringa* and found 512 total polymorphic fragments with 372 useful for analyzing genome variation among species.

Microsatellite markers (SSRs) are useful co-dominant markers that are sometimes transferrable to other genera within a family (Yashoda et al., 2005; White and Powell, 1997; Barreneche et al., 2004). De La Rosa et al. (2002) and Lendvay et al. (2013) demonstrated that previously reported marker data from closely related genera can prove useful for marker development in *Syringa*. Closely related genera within Oleaceae (Wallander and Albert, 2000) have potentially useful published markers, include taxa such as *Phillyrea* (Saumitou-Laprade et al., 2000), *Olea* (Beghe et al., 2011; Carriero et al., 2002; Cipriani et al., 2002; Corrado et al., 2011; Ercisli et al., 2011; Gil et al., 2006; Gismondi and Canini, 2013, Rallo et al., 2000,2003; Rotondi et al., 2011), *Osmanthus* (Zhang et al., 2011), *Ligustrum* (Kodama et al., 2008), *Fraxinus* (Brachet et al., 1999; Lefort et al., 1999; Lukšienė et al., 2012; Verdú et al., 2004, 2006) and *Chionanthus* (Arias et al., 2011; Rinehart/Olsen - Unpublished). Though previously published data may prove useful for identifying

polymorphic markers in *Syringa*, novel markers may be economically discovered by the advent of next generation sequencing.

Next-generation sequencing (NGS), with Illumina® being the most widely used platform, has become a useful tool in generating high-throughput, massively parallel sequence data at a low cost compared to traditional Sanger sequencing (Egan et al., 2012). Sequence data generated from NGS can be useful in discovering SSRs in minor horticultural crops that lack published marker information (Jennings et al. 2011). In addition to deep sequencing, genotyping-by-sequencing of coding regions can be valuable for producing thousands of SNP markers for genetic mapping. Genetic linkage maps can be constructed based on recombination frequency and show the relative positions of multiple marker types. For wide crosses between heterozygous parents that produce highly polymorphic F_1 mapping populations, a double pseudo-testcross strategy was proposed (Grattapaglia and Sederoff, 1994). This method has successfully produced independent parental maps in minor taxa such as *Eucalyptus* (Grattapaglia and Sederoff, 1994), *Morus* (Venkateswarlu et al., 2006), *Vitis* (Lowe and Walker, 2006), and *Dendrobium* (Feng, et al., 2013).

The objectives of this study were to 1) create a bi-parental mapping population segregating for disease resistance and remontancy, and 2) construct preliminary, SNP-based genetic linkage maps of *S. meyeri* ‘Palibin’ and *S. pubescens* Bloomerang® using the double pseudo-testcross method.

Methods and Materials

Plant Materials. To create the bi-parental mapping population, the female parent *S. meyeri* ‘Palibin’ (10-0209) was acquired from Blue Heron Nursery

(Corvallis, OR). Two "clones" of the male parent were collected. The first was *S. pubescens* 'Penda' Bloomerang® Purple (12-0026) acquired from Garland Nursery (Corvallis, OR). This parent was used for crosses in 2012 and 2013. The second was *S. pubescens* Bloomerang® (13-0070) acquired from Monrovia Nursery (Dayton, OR) which was used for crosses in 2013. The reported female parent of Bloomerang®, Josee™ (10-0039), was acquired from Blue Heron Nursery and was also included in the mapping population for future studies. The parent materials and resulting progeny were maintained at the Lewis Brown Horticulture Farm at Oregon State University (Corvallis, OR).

Development of mapping population. During the spring of 2012 and 2013, fresh pollen was collected from Bloomerang® and stored in small petri dishes over desiccant (Drierite; W.A. Hammond Drierite, Xenia, OH) in a refrigerator at 4 ˚C. Prior to pollination, open flowers were removed on 'Palibin' and the plant was placed in a glasshouse free of pollinators with day/night temperatures of 25/20 °C and a 16-h photoperiod. Unopened flowers were emasculated and each flower was pollinated using a small paintbrush two or three times post emasculation over consecutive days. Developing fruit were observed though the spring and summer, and dry dehiscent capsules were collected in the late summer. Seeds were placed into plastic bags containing a 1:1 stratification mix of perlite (Supreme Perlite Co., Portland, OR) and growing medium (Metro-Mix; Sun Gro Horticulture, Agawam, MA). Seeds were cold-stratified for 10 weeks at 4 °C and sown in 1.3-L containers filled with growing medium at approximately 30 seeds per pot. After sowing in containers, seeds were treated once with copper hydroxide (Kocide 2000; DuPont, Wilmington, DE) at 0.3

$mg \cdot L^{-1}$ to reduce fungal contamination of germinating seeds. A total of 416 seedlings were recovered over two years of crosses between 'Palibin' and Bloomerang®. Seedlings were grown in containers from 2013-2017, and mature plants were field-planted in 2017 at the Lewis Brown Horticulture Farm for future phenotyping.

DNA extraction and library preparation. Leaf samples were collected in the spring of 2015 as young leaves began to emerge. Approximately 50 mg of leaf tissue was collected for each sample, placed in plates of microtubes (10 × 96 Collection Microtubes #19560; Qiagen, Hilden, Germany) and kept over ice while being transported to the laboratory. The female parent, 'Palibin' was replicated six times through the sampling population (Table 5.1). The two male parents, Bloomerang®, were each replicated three times throughout the sampling population, for a total of six replicates for Bloomerang® (Table 5.1). The reported female parent of Bloomerang®, *S. pubescens* Josee™ (10-0039), was also replicated six times throughout the sampling population (Table 5.1). Samples were submitted to the Center for Genome Research and Biocomputing (CGRB) at Oregon State University (Corvallis, OR) for DNA extraction, quantification, and genotyping-by-sequencing (GBS) library preparation.

Samples were stored at -80 °C until DNA extraction. DNA extraction was performed using an automated extraction system (Thermo KingFisher Flex; Thermo Fisher Scientific, Waltham, MA). GBS library preparation was performed using the titration methods, barcodes, adapters and primers described by Elshire et al. (2011). Briefly, eight titrations were used to optimize adapter concentrations, as not to introduce an excess of adapters and waste sequencing reads. Plates of DNA were

normalized to 100 ng per individual prior to library preparation. Enzymatic digestion was performed with *Ape*KI (New England Biolabs, Ipswich, MA, USA), fragments were ligated to common and unique barcodes using T4 ligase (New England Biolabs), and individuals were pooled (Elshire et al., 2011). Prior to quantitation, the ligation reaction was purified using spin column and PCR cleanup kits (Quiagen; Valencia, CA). Quantitation was performed using a Qubit® fluorometer (Invitrogen; Carlsbad, CA). For library size distribution confirmation, DNA was tested using the Bioanalyzer 2100 HS-DNA chip (Agilent Technologies; Santa Clara, CA). GBS libraries were diluted and submitted for further quantification using quantitative PCR (qPCR) to allow for accurate loading in the Illumina flowcell and for good cluster generation. DNA from pooled libraries was loaded for paired-end sequencing performed using three lanes of the Illumina HiSeq 3000 platform (Illumina Biotechnology; San Diego, CA). Each lane included 42 individuals from the F_1 population, two reps of 'Palibin', two reps of Josee™, one rep of each "clone" of Bloomerang®.

SNP calling and linkage analysis. SNP calling was performed using the TASSLE GBS discovery software pipeline (Li et al., 2009). Haplotype maps (HapMaps) were constructed using the TASSEL UNEAK pipeline provided by the Buckler Lab for Maize Genetics and Diversity. Initial SNP filtering was performed in Excel (Microsoft; Redmond, WA). Linkage map construction and analysis was performed in Joinmap v4.1 (Van Ooijen, 2006) and visualization was performed in MapChart 2.3 (Voorips, 2002).

Results and Discussion

SNP filtering. After HapMaps were combined using the TASSLE UNEAK pipeline, SNPs were analyzed and filtered in Excel prior to importing into JoinMap. A total of 20,729 SNPs were discovered with the largest percent missing value at 19% across progeny. SNP calls on Bloomerang® revealed high levels of heterozygosity between the two "clones" used to create the mapping population. Therefore, only progeny created from crosses using the original Bloomerang® (12-0026) "clone" were investigated further. In addition, 13 of these original seedlings were later discovered to be reciprocal crosses (Bloomerang® x 'Palibin') and were excluded (Table 5.1). Therefore, 79 progeny in total were used for SNP filtering and linkage analysis (Table 5.1).

For each SNP marker, columns (Excel) representing genotype totals were constructed, as well as columns for percent missing values. For each marker, a χ^2 goodness-of-fit test was performed to reveal markers segregating 1 : 1 (homozygous : heterozygous or heterozygous : homozygous) or 1 : 2 : 1 (homozygous : heterozygous : homozygous). Any χ^2 test with a $P < 0.1$ was discarded. Based on the female and male genotypes, data were separated and further SNP filtering was performed. Markers were eliminated if they had any ambiguity in parent SNP calls. For 1 : 1 segregating SNPs, occasionally seedlings would have SNP calls that did not fit the parent genotypes. If more than five progeny produced these misreads, the marker was eliminated. If five or less progeny produced these misreads, then misreads were changed to missing values. Included with the nearly 7,000 candidate 1 : 1 markers, the top 1,000 1 : 2 : 1 markers (based on χ^2 test) were included with

each set of parental 1 : 1 makers. The total set of markers used to construct the linkage maps represented 31% of the original 20,729 SNP markers discovered from the SNP-calling pipeline.

Construction of genetic linkage map. Linkage maps for each parent were constructed independently using the double pseudo-testcross method described by Grattapaglia and Sederoff (1994). Prior to importing SNP markers into JoinMap, all SNPs were recoded as lm x ll (female heterozygous and male homozygous), nn x np (female homozygous and male heterozygous) or hk x hk (female heterozygous and male heterozygous). Each data set was imported separately and used to create population nodes for further filtering. For each data set, some markers mapped to the same or similar location. Using a calculation for the similarity of loci, redundant markers were removed if they had > 97% similarity with another SNP marker. Next, groupings trees for set of parental markers were generated based on a maximum likelihood mapping algorithm. Potential linkage groups were selected from the grouping trees based on large groups of linked loci that remained linked at a logarithm of odds (LOD) score of 10. For the female map constructed of lm x ll and hk x hk markers, 22 linkage groups were selected. For the male map, 25 linkage groups were selected. Both of these groupings fall near the true haploid chromosome number for lilacs of $x = 23$.

A linkage map was generated for each linkage group for the female and male parents. Suspect linkages that caused >20 cM gaps near the ends of linkage groups were removed. Suspect linkages that caused >20 cM gaps within the linkage groups were not removed from the preliminary map. Mapping issues may be due to lilac's

large genome, which has likely developed over successive rounds of polyploidy followed by diploidization. Future sequencing and marker discovery may rectify suspect linkages of the preliminary linkage maps. In total, a relatively small number of markers were removed across all linkage groups in the female and male maps. The resulting parental maps were constructed of linkage groups that varied in size from 76.5 cM to 1143.5 cM (Table 5.2).

Based on flow cytometry estimates of Bloomerang®, lilac has a large genome at nearly 1300 Mbp. For the female parent, 'Palibin', the preliminary map was 11,492 cM long over 22 linkage groups (Table 5.2). The total number of SNP markers used to construct the female map was 3,554 with an average map distance between markers of 3.4 cM (Table 5.2). The number of markers of lm x ll markers mapped to 'Palibin' totaled 2,665 while the number of hk x hk markers totaled 889 (Table 5.2). Although a concensus map was not able to be constructed, hk x hk markers cosegregating in each parent were able connect 20 out of the 22 linkage groups in 'Palabin' to linkage groups in the male linkage map (Fig. 5.1). Only linkage group P18 and P20 were unable to be combined with their corresponding linkage groups in the male map (Fig. 5.1). Only three hk x hk markers mapped to linkage group P18 while linkage group P20 consisted only of hk x hk markers. Several linkage groups in the female map were much longer than their corresponding linkage groups in the male map. The longest linkage group, P8, at over 1,000 cM mapped to two different linkage groups in the male map, B7 and B8 (Fig. 5.1). Therefore, P8 is likely two different linkage groups, and future revisions to the preliminary map will likely resolve suspect linkages in P8.

For the male parent, Bloomerang®, the preliminary map was 8,717 cM long over 25 linkage groups (Table 5.2). The male map was much smaller than the female, with more linkage groups present (Fig. 5.1). The total number of SNP markers used to construct the male map was 2,925 with an average map distance between markers of 3.2 cM (Table 5.2). The number of nn x np markers mapped to Bloomerang® totaled 2,034 while the number of hk x hk markers totaled 891 (Table 5.2). Although a concensus map was not able to be constructed, hk x hk markers cosegregating in each parent were able to connect 22 out of the 25 linkage groups in Bloomerang® to linkage groups in the female linkage map (Fig. 5.1). Linkage groups B6, B23, and B24 were unable to be combined with their corresponding linkage groups in the female linakage map (Fig. 5.1). Only 18 markers mapped to the minor linkage group, B6, with no hk x hk markers present (Table 5.2). A total of 47 markers mapped to B23, with no hk x hk markers present (Table 5.2). A total of 46 markers mapped to B24 with only two hk x hk markers present (Table 5.2).

Few genetic linkage maps exist for economically important ornamental crops, which are often complicated by polyploidy and large genomes. In our study, we report the first preliminary genetic linkage maps for dwarf lilacs. This group of lilacs exhibits important variable traits such as remontancy and disease resistance, which often take many years to express in breeding populations. Creating a genetic linkage map in lilac is complicated by the lack of a draft genome to aid alignment of sequence data, and by its large genome at approximately 1300 Mbp. Although the preliminary linkage maps in dwarf lilac were densly populated in the current study, further work must be performed to further improve mapping as well as construct a consensus map.

Deep sequencing has recently been performed on the male parent, Bloomerang®, of the bi-parental mapping population. In addition, phenotype data will be collected on the mapping population over several years as plants mature for use in future marker-trait association mapping. As phenotype data is collected, future work will focus on construcing a draft genome for Bloomerang® for alignment of the GBS data as well as discovery of repeat motifs for constructing SSR primers. Early phenotypic observations have revealed that the mapping population is beginning to segregate for remontancy as well as resistance to bacterial blight. As the preliminary maps improve and as phenotype data is collected, we continue to improve the prospects of perfoming marker-assisted selection in this important group of ornamental landscape shrubs.

Literature Cited

Arias, R.S., N. Techen, T.A. Rinehart, R.T. Olsen, J.H. Kirkbride Jr. and B.E. Scheffler. 2011. Development of simple sequence repeat markers for *Chionanthus retusus* (Oleaceae) and effective discrimination of closely related taxa. HortScience 46: 23-29.

Barreneche, T., M. Casasoli, K. Russell, A. Akkak, H. Meddour, C. Plomion, F. Villani and A. Kremer. 2004. Comparative mapping between *Quercus* and *Castanea* using simple-sequence repeats (SSRs). Theor. Appl. Genet. 108:558-566.

Beghe, D., A. Ferrarini, T. Ganino and A. Fabbri. 2011. Molecular characterization and identification of a group of local *Olea europaea* L. varieties. Tree Genet. Genomes 7:1185-1198.

Brachet, S., M.F. Jubier, M. Richard, B. Jung-Muller and N. Frascaria-Lacoste. 1999. Rapid identification of microsatellite loci using 5' anchored PCR in the common ash *Fraxinus excelsior*. Molecular Ecology 8: 160-163.

Bushakra, J.M., M.J. Stephens, A.N. Atmadjaja, K.S. Lewers, V.V. Symonds, J.A. Udall, D. Chagné, E.J. Buck and S.E. Gardiner. 2011. Construction of black (*Rubus occidentalis*) and red (*R. idaeus*) raspberry linkage maps and their comparison to the genomes of strawberry, apple, and peach. Theor. Appl. Genet. 125:311-327.

Carriero, F., G. Fontanazza, F. Cellini and G. Giorio. 2002. Identification of simple sequence repeats (SSRs) in olive (*Olea europaea* L.). Theor. Appl. Genet. 104:301-307.

Cipriani, G., M.T. Marrazzo, R. Marconi, A. Cimato and R. Testolin. 2002. Microsatellite markers isolated in olive (*Olea europaea* L.) are suitable for individual fingerprinting and reveal polymorphism within ancient cultivars. Theor. Appl. Genet. 104:223-228.

Corrado, G., A. Imperato, M. La Mura, E. Perri and R. Rao. 2011. Genetic diversity among olive varieties of Southern Italy and the traceability of olive oil using SSR markers. J. Hortic. Sci. Biotechnol. 86:461-466.

Darlington, C.D. and A.P. Wylie. 1956. Chromosome atlas of flowering plants. Macmillan, New York, NY.

De La Rosa, R., C.M. James and K.R. Tobutt. 2002. Isolation and characterization of polymorphic microsatellites in olive (*Olea europaea* L.) and their transferability to other genera in the Oleaceae. Molecular Ecology Notes 2:265-267.

Egan, A.N., J. Schlueter and D.M. Spooner. Applications of Next-Generation Sequencing in Plant Biology. American Journal of Botany 99:175-185.

Elshire, R.J., J.C. Glaubitz, Q. Sun, J. Poland, K. Kawamoto, and E.S. Buckler. 2011. A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. PLoS One 6: e19379. 10.1371/journal.pone.0019379.g006.

Ercisli, S., A. Ipek and E. Barut. 2011. SSR marker-based DNA fingerprinting and cultivar identification of olives (*Olea europaea*). Biochem. Genet. 49:555-561.

Feng, S., H. Zhao, J. Lu, J. Liu, B. Shen, and H. Wang. 2013. Preliminary genetic linkage maps of Chinese herb *Dendrobium nobile* and *D. moniliforme*. Journal of Genetics 92:205-212.

Gil, F. Sabino, M. Busconi, A. Da Câmara Machado and C. Fogher. 2006. Development and characterization of microsatellite loci from *Olea europaea*. Molecular Ecology Notes 6:1275-1277.

Gilmore, B., N. Bassil, A. Nyberg, B. Knaus, D. Smith, D.L. Barney and K. Hummer. 2013. Microsatellite Marker Development in Peony using Next Generation Sequencing. J. Amer. Soc. Hort. Sci. 138:64-74.

Gilmore, B.S., N.V. Bassil, D.L. Barney, B.J. Knaus and K.E. Hummer. 2014. Short-read DNA Sequencing Yields Microsatellite Markers for *Rheum*. J. Amer. Hort. Sci. 139:22-29.

Gismondi, A. and A. Canini. 2013. Microsatellite analysis of Latial *Olea europaea* L. cultivars. Plant Biosystems 147:686-691.

Grattapaglia, D. and R. Sederoff. 1994. Genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* using a pseudo-testcross: mapping strategy and RAPD markers. Genetics 137:1121-1137.

Jennings, T., B. Knaus, T. Mullins, S. Haig and R. Cronn. 2011. Multiplexed microsatellite recovery using massively parallel sequencing. Mol. Ecol. Notes 11:1060-1067.

Juntheikki-Palovaara, I., K. Antonius, L. Lindén and H. Korpelainen. 2013. Microsatellite markers for common lilac (*Syringa vulgaris* L.). Plant Genetic Resources: Characterization and Utilization 11:279-282.

Kim, K.-J. and R.K. Jansen. 1998. A chloroplast DNA phylogeny of lilacs (*Syringa*, Oleaceae): plastome groups show a strong correlation with crossing groups. American Journal of Botany 85: 1338-1351.

Kodama, K., T. Yamada and M. Maki. 2008. Development and characterization of 10 microsatellite markers for the semi-evergreen tree species, *Ligustrum ovalifolium* (Oleaceae). Molecular Ecology Resources 8:1008-1010.

Kochieva, E.Z., N.N. Ryzhova, O.I. Molkanova, A.M. Kudryavtsev, V.P. Upelniek and I.B. Okuneva. 2004. The Genus *Syringa*: Molecular Markers of Species and Cultivars. Russian Journal of Genetics 40: 30-32.

Lattier, J.D. and R.N. Contreras. 2017. Intraspecific, interspecific, and interseries cross-compatibility in lilac. J. Amer. Soc. Hort. Sci. 142: 279-288.

Lefort, F., S. Brachet, N. Frascaria-Lacoste, K.J. Edwards and G.C. Douglas. 1999. Identification and characterization of microsatellite loci in ash (*Fraxinus excelsior* L.) and their conservation in the olive family (Oleaceae). Molecular Ecology 8:1088-1089.

Lendvay, B., A. Pedryc and M Höhn. 2013. Characterization of Nuclear Microsatellite Markers for the Narrow Endemic *Syringa josikaea* Jacq. Fil. Ex Rchb. Not. Bot. Horti. Agrobo. 41:301-305.

Li, H. B. Handsaker, A. Wysoker, T. Fennel, J. Ruan, N. Homer, G. Marth, G. Abecasis, R. Durbin, Subgroup GPDP. 2009. The sequence alignment/map format and SAMtools. Bioinformatics 25:2078-2079.

Li, J., B. Goldman-Huertas, J. DeYoug and J. Alexander, III. 2012. Phylogenetics and diversification of *Syringa* inferred from nuclear and plastid DNA sequences. Castanea 77: 82-88.

Lowe, K.M. and M.A. Walker. 2006. Genetic linkage map of the interspecific grape rootstock cross Ramsey (*Vitis champinii*) × Riparia Gloire (*Vitis riparia*). Theor. Appl. Genet. 112:1582-1592.

Lukšienė, R., B. Heinze and S. Kuusienė. 2012. Evaluation of Genetic Diversity in Selected Fraxinus excelsior L. Trees and Their Offspring using RAPD and Microsatellite Methods. Baltic Forestry 18:65-72.

Mmbaga, M.T., A. Shi, and M.-S. Kim. 2011. Identification of *Alternaria alternata* as causal agent for leaf blight in *Syringa* species. Plant Pathology J. 27:120-127.

Mmbaga, M.T. and H. Sheng. 1997. Evaluation of lilac (*Syringa* spp.) for multiple disease resistance to powdery mildew and bacterial blight in McMinnville, TN. Proceedings of the Southern Nurseryman's Association Research Conference 42:512-518.

Mmbaga, M.T., R.J. Suavé, E. Nnodu, and S. Zhou. 2005. Multiple disease resistance to powdery mildew, bacterial blight, and *Alternaria* blight in lilac (*Syringa* spp.). J. Arboriculture. 31:1-9.

Rallo, P., G. Dorado and A. Martín. 2000. Development of simple sequence repeats in olive tree (*Olea europaea* L.) Theor. Appl. Genet. 101:984-989.

Rallo, P., I. Tenzer, C. Gessler, L. Baldoni, G. Dorado and A. Martín. 2003. Transferability of olive microsatellite loci across the genus *Olea*. Theor. Appl. Genet. 107:940-946.

Rotondi, A., N.G.M. Cultrera, R. Mariotti and L. Baldoni. 2011. Genotyping and evaluation of local olive varieties of a climatically disfavoured region through molecular, morphologically and oil quality parameters. Sci. Hortic. 130:562-569.

Rzepka-Plevneš, D., M. Smolik and K. Tańska. 2006. Genetic similarity of chosen *Syringa* species determined by the ISSR-PCR technique. Dendrobiology 56:61-67.

Saumitou-Laprade, P., C. Vassiliadis, J.T. Epplen and C. Hardt. 2000. Molecular Ecology 9:107-118.

Sax, K. 1930. Chromosome number and behavior in the genus Syringa. Journal of the Arnold Arboretum 11:7-14.

Schuelke, M. 2000. An economic method for the fluorescent labeling of PCR fragments. Nat. Biotechnol. 18:233-234.

Stebbins, Jr., G.L. 1940. The significance of polyploidy in plant evolution. Amer. Naturalist 74:54-66.

Taylor, H. 1945. Cyto-taxonomy and phylogeny of the Oleaceae. Brittonia 5:337-367.

Van Ooijen, J.W. 2006. JoinMap® 4, software for the calculation of genetic linkage maps in experimental populations. Kyazma B.V., Wageningen.

Verdú, M., A.I. Montilla and J.R. Pannell. 2004. Paternal effects on functional gender account for cryptic dioecy in a perennial plant. Proc. R. Soc. Lond. B 271:2017-2023.

Venkateswarlu M., S.R. Urs, B.S. Nath, H.E. Shashidhar, M. Maheswaran, T.M. Veeraiah and M.G. Sabitha. 2006. A first genetic linkage map of mulberry

(*Morus* spp.) using RAPD, ISSR, and SSR markers and pseudotestcross mapping strategy. Tree Genet. Genomes 3, 15–24.

Verdú, M., S.C. Gonzáles, A.I. Montilla, I. Mateu and J.R. Pannell. 2006. Ovule discounting in an outcrossing, cryptically dioecious tree. Evolution 60:2056-2063.

Voorrips, RE (2002) MapChart: software for the graphical presentation of linkage maps and QTLs. J Hered 93:77–78.

Wallander, E. and V.A. Albert. 2000. Phylogeny and Classification of Oleaceae Based on *RPS16* and *TRNL-F* Sequence Data. American Journal of Botany 87:1827-1841.

White, G. and W. Powell. 1997. Cross-species amplification of SSR loci in the Meliaceae family. Molecular Ecology 6:1195-1197.

Yasodha, R., M. Ghosh, R. Sumathi and K. Gurumurthi. 2005. Cross-species amplification of eucalyptus SSR markers in Casuarinaceae. Acta Botanica Croatica 64: 115-120.

You, F., N. Huo, Y. Gu, M. Luo, Y. Ma, D. Hane, G. Lazo, J. Dvorak and O. Anderson. 2008. Batchprimer3: A high throughput web application for PCR and sequencing primer design. BMC Bioinformatics 9:253.

Zhang, Z.-R., D.-M. Fan, S.-Q. Guo, D.-Z. Li and Z.-Y. Zang. 2011. Development of 29 microsatellite markers for *Osmanthus fragrans* (Oleaceae), a traditional fragrant flowering tree of China. American Journal of Botany 98:e356-e359.

Tables

Table 5.1. Lilac leaf samples collected for genotyping-by-sequencing.

Plate no.[z]	Well[y]	Sample name[x]	Accession no.[w]
Plate 1	A1	Palabin*	10-0209
Plate 1	B1	Josee	10-0039
Plate 1	C1	Bloomerang 1*	12-0026
Plate 1	D1	JL001	BxP H2012-044-004
Plate 1	E1	JL002	BxP H2012-044-018
Plate 1	F1	JL003	BxP H2012-044-017
Plate 1	G1	JL004	BxP H2012-044-016
Plate 1	H1	JL005	BxP H2012-044-015
Plate 1	A2	JL006	BxP H2012-044-012
Plate 1	B2	JL007	BxP H2012-044-003
Plate 1	C2	JL008	BxP H2012-044-001
Plate 1	D2	JL009	BxP H2012-044-010
Plate 1	E2	JL010	BxP H2012-044-008
Plate 1	F2	JL011	BxP H2012-044-005
Plate 1	G2	JL012	BxP H2012-044-002
Plate 1	H2	JL013	BxP H2012-044-014
Plate 1	A3	Palabin*	10-0209
Plate 1	B3	Josee	10-0039
Plate 1	C3	Bloomerang 2	13-0070
Plate 1	D3	JL014*	PxB H2012-043-062
Plate 1	E3	JL015*	PxB H2012-043-043
Plate 1	F3	JL016*	PxB H2012-043-063
Plate 1	G3	JL017*	PxB H2012-043-077
Plate 1	H3	JL018*	PxB H2012-043-079
Plate 1	A4	JL019*	PxB H2012-043-076
Plate 1	B4	JL020*	PxB H2012-043-081
Plate 1	C4	JL021*	PxB H2012-043-075
Plate 1	D4	JL022*	BxP H2012-044-013
Plate 1	E4	JL023*	PxB H2012-043-056
Plate 1	F4	JL024*	PxB H2012-043-072
Plate 1	G4	JL025*	PxB H2012-043-057
Plate 1	H4	JL026*	PxB H2012-043-069
Plate 1	A5	JL027*	PxB H2012-043-070

Table 5.1 (continued). Lilac leaf samples collected for genotyping-by-sequencing.

Plate no.	Well	Sample name	Accession no.
Plate 1	B5	JL028*	PxB H2012-043-058
Plate 1	C5	JL029*	PxB H2012-043-073
Plate 1	D5	JL030*	PxB H2012-043-059
Plate 1	E5	JL031*	PxB H2012-043-060
Plate 1	F5	JL032*	PxB H2012-043-074
Plate 1	G5	JL033*	PxB H2012-043-071
Plate 1	H5	JL034*	PxB H2012-043-061
Plate 1	A6	JL035*	PxB H2012-043-076
Plate 1	B6	JL036*	PxB H2012-043-080
Plate 1	C6	JL037*	PxB H2012-043-082
Plate 1	D6	JL038*	PxB H2012-043-064
Plate 1	E6	JL039*	PxB H2012-043-019
Plate 1	F6	JL040*	PxB H2012-043-038
Plate 1	G6	JL041*	PxB H2012-043-048
Plate 1	H6	JL042*	PxB H2012-043-049
Plate 1	A7	Palabin*	10-0209
Plate 1	B7	Josee	10-0039
Plate 1	C7	Bloomerang 1*	12-0026
Plate 1	D7	JL043*	PxB H2012-043-006
Plate 1	E7	JL044*	PxB H2012-043-035
Plate 1	F7	JL045*	PxB H2012-043-012
Plate 1	G7	JL046*	PxB H2012-043-036
Plate 1	H7	JL047*	PxB H2012-043-034
Plate 1	A8	JL048*	PxB H2012-043-014
Plate 1	B8	JL049*	PxB H2012-043-033
Plate 1	C8	JL050*	PxB H2012-043-042
Plate 1	D8	JL051*	PxB H2012-043-044
Plate 1	E8	JL052*	PxB H2012-043-045
Plate 1	F8	JL053*	PxB H2012-043-065
Plate 1	G8	JL054*	PxB H2012-043-022
Plate 1	H8	JL055*	PxB H2012-043-066
Plate 1	A9	Palabin*	10-0209
Plate 1	B9	Josee	10-0039
Plate 1	C9	Bloomerang 2	13-0070
Plate 1	D9	JL056*	PxB H2012-043-067
Plate 1	E9	JL057*	PxB H2012-043-037
Plate 1	F9	JL058*	PxB H2012-043-030

Table 5.1 (continued). Lilac leaf samples collected for genotyping-by-sequencing.

Plate no.	Well	Sample name	Accession no.
Plate 1	G9	JL059*	PxB H2012-043-051
Plate 1	H9	JL060*	PxB H2012-043-010
Plate 1	A10	JL061*	PxB H2012-043-053
Plate 1	B10	JL062*	PxB H2012-043-050
Plate 1	C10	JL063*	PxB H2012-043-028
Plate 1	D10	JL064*	PxB H2012-043-013
Plate 1	E10	JL065*	PxB H2012-043-015
Plate 1	F10	JL066*	PxB H2012-043-017
Plate 1	G10	JL067*	PxB H2012-043-039
Plate 1	H10	JL068*	PxB H2012-043-016
Plate 1	A11	JL069*	PxB H2012-043-020
Plate 1	B11	JL070*	PxB H2012-043-023
Plate 1	C11	JL071*	PxB H2012-043-024
Plate 1	D11	JL072*	PxB H2012-043-025
Plate 1	E11	JL073*	PxB H2012-043-068
Plate 1	F11	JL074*	PxB H2012-043-047
Plate 1	G11	JL075*	PxB H2012-043-007
Plate 1	H11	JL076*	PxB H2012-043-009
Plate 1	A12	JL077*	PxB H2012-043-001
Plate 1	B12	JL078*	PxB H2012-043-002
Plate 1	C12	JL079*	PxB H2012-043-004
Plate 1	D12	JL080*	PxB H2012-043-005
Plate 1	E12	JL081*	PxB H2012-043-032
Plate 1	F12	JL082*	PxB H2012-043-003
Plate 1	G12	JL083*	PxB H2012-043-018
Plate 1	H12	JL084*	PxB H2012-043-055
Plate 2	A1	Palabin*	10-0209
Plate 2	B1	Josee	10-0039
Plate 2	C1	Bloomerang 1*	12-0026
Plate 2	D1	JL085*	PxB H2012-043-040
Plate 2	E1	JL086*	PxB H2012-043-041
Plate 2	F1	JL087*	PxB H2012-043-027
Plate 2	G1	JL088*	PxB H2012-043-021
Plate 2	H1	JL089*	PxB H2012-043-054
Plate 2	A2	JL090*	PxB H2012-043-026
Plate 2	B2	JL091*	PxB H2012-043-011
Plate 2	C2	JL092*	PxB H2012-043-008

Table 5.1(continued). Lilac leaf samples collected for genotyping-by-sequencing.

Plate no.	Well	Sample name	Accession no.
Plate 2	D2	JL093	PxB H2013-156-296
Plate 2	E2	JL094	PxB H2013-156-118
Plate 2	F2	JL095	PxB H2013-156-127
Plate 2	G2	JL096	PxB H2013-156-200
Plate 2	H2	JL097	PxB H2013-156-185
Plate 2	A3	Palabin	10-0209
Plate 2	B3	Josee	10-0039
Plate 2	C3	Bloomerang 2	13-0070
Plate 2	D3	JL098	PxB H2013-156-009
Plate 2	E3	JL099	PxB H2013-156-039
Plate 2	F3	JL100	PxB H2013-156-063
Plate 2	G3	JL101	PxB H2013-156-008
Plate 2	H3	JL102	PxB H2013-156-071
Plate 2	A4	JL103	PxB H2013-156-031
Plate 2	B4	JL104	PxB H2013-156-129
Plate 2	C4	JL105	PxB H2013-156-125
Plate 2	D4	JL106	PxB H2013-156-104
Plate 2	E4	JL107	PxB H2013-156-172
Plate 2	F4	JL108	PxB H2013-156-116
Plate 2	G4	JL109	PxB H2013-156-105
Plate 2	H4	JL110	PxB H2013-156-007
Plate 2	A5	JL111	PxB H2013-156-002
Plate 2	B5	JL112	PxB H2013-156-123
Plate 2	C5	JL113	PxB H2013-156-075
Plate 2	D5	JL114	PxB H2013-156-010
Plate 2	E5	JL115	PxB H2013-156-091
Plate 2	F5	JL116	PxB H2013-156-092
Plate 2	G5	JL117	PxB H2013-156-096
Plate 2	H5	JL118	PxB H2013-156-018
Plate 2	A6	JL119	PxB H2013-156-042
Plate 2	B6	JL120	PxB H2013-156-047
Plate 2	C6	JL121	PxB H2013-156-067
Plate 2	D6	JL122	PxB H2013-156-046
Plate 2	E6	JL123	PxB H2013-156-121
Plate 2	F6	JL124	PxB H2013-156-062
Plate 2	G6	JL125	PxB H2013-156-056
Plate 2	H6	JL126	PxB H2013-156-167

[z]Samples collected in two 96-well plates.
[y]Position in 96-well plate.
[x]Designated GBS sample name. * = included in genetic linkage map.
[w]Plant accession number in ornamental breeding program (Oregon State University, Corvallis, Oregon). Cross Bloomerang® x 'Palabin' abbreviated = BxP. 'Palabin' x Bloomerang® = PxB.

Table 5.2. Number, size, and SNP maker density of linkage groups for female and male parents of a lilac bi-parental mapping population.

S. meyeri 'Palabin'[z]						*S. pubescens* Bloomerang®[y]					
Linkage group	lm x ll makers (no.)[x]	hk x hk markers (no.)[w]	Total markers (no.)	Size (cM)[v]	Avg. marker distance (cM)[u]	Linkage group	lm x ll makers (no.)[x]	hk x hk markers (no.)[w]	Total markers (no.)	Size (cM)[v]	Avg. marker distance (cM)[u]
P1	83	199	282	801.4	2.9	B1	55	200	255	571.5	2.9
P2	172	15	187	536.6	2.9	B2	114	25	139	364.1	2.6
P3	129	6	135	585.1	4.4	B3	119	97	216	541.1	2.5
P4	113	20	133	420.6	3.2	B4	143	59	202	527.1	2.6
P5	160	97	257	690.9	2.7	B5	77	54	131	322.2	2.6
P6	107	25	132	390.9	3.0	B6	18	0	18	76.5	4.5
P7	90	15	105	357.0	3.4	B7	41	54	95	253.6	2.7
P8	248	41	289	1143.5	4.0	B8	15	49	64	304.0	4.8
P9	205	27	232	615.6	2.7	B9	136	15	151	471.1	3.1
P10	94	102	196	699.4	3.6	B10	95	43	138	432.4	3.2
P11	136	59	195	595.4	3.1	B11	110	26	136	354.9	2.6
P12	124	26	150	376.2	2.5	B12	118	15	133	556.6	4.2
P13	118	27	145	456.7	3.2	B13	85	30	115	357.6	3.1
P14	105	43	148	558.7	3.8	B14	79	20	99	306.0	3.1
P15	87	54	141	399.5	2.9	B15	68	19	87	274.8	3.2
P16	96	40	136	408.6	3.0	B16	216	28	244	653.7	2.7
P17	106	19	125	491.7	4.0	B17	90	40	130	442.6	3.4
P18	111	4	115	513.7	4.5	B18	129	7	136	502.6	3.7
P19	69	26	95	258.6	2.8	B19	70	20	90	366.6	4.1
P20	0	14	14	62.9	4.8	B20	68	6	74	194.4	2.7
P21	215	10	225	758.5	3.4	B21	33	42	75	179.4	2.4
P22	97	20	117	370.6	3.2	B22	29	30	59	165.4	2.9
						B23	47	0	47	192.9	4.2
						B24	44	2	46	178.8	4.0
						B25	35	10	45	127.2	2.9

[z]Female parent of bi-parental mapping population: *Syringa meyeri* 'Palabin'.
[y]Male parent of bi-parental mapping parent: Syringa pubescens 'Penda' Bloomerang® purple.
[x]Number of 1:1 segregating SNP markers coded for JoinMap 4.1. Female markers: lm x ll. Male markers nn x np.
[w]Numer of 1:2:1 segregating SNP markers coded for JoinMap 4.1. Shared female and male markers segregating in both parents: hk x hk.
[v]Length of each linkage group in centi-Morgans.
[u]Average distance in centi-Morgans between each marker on each linkage group.

Figures

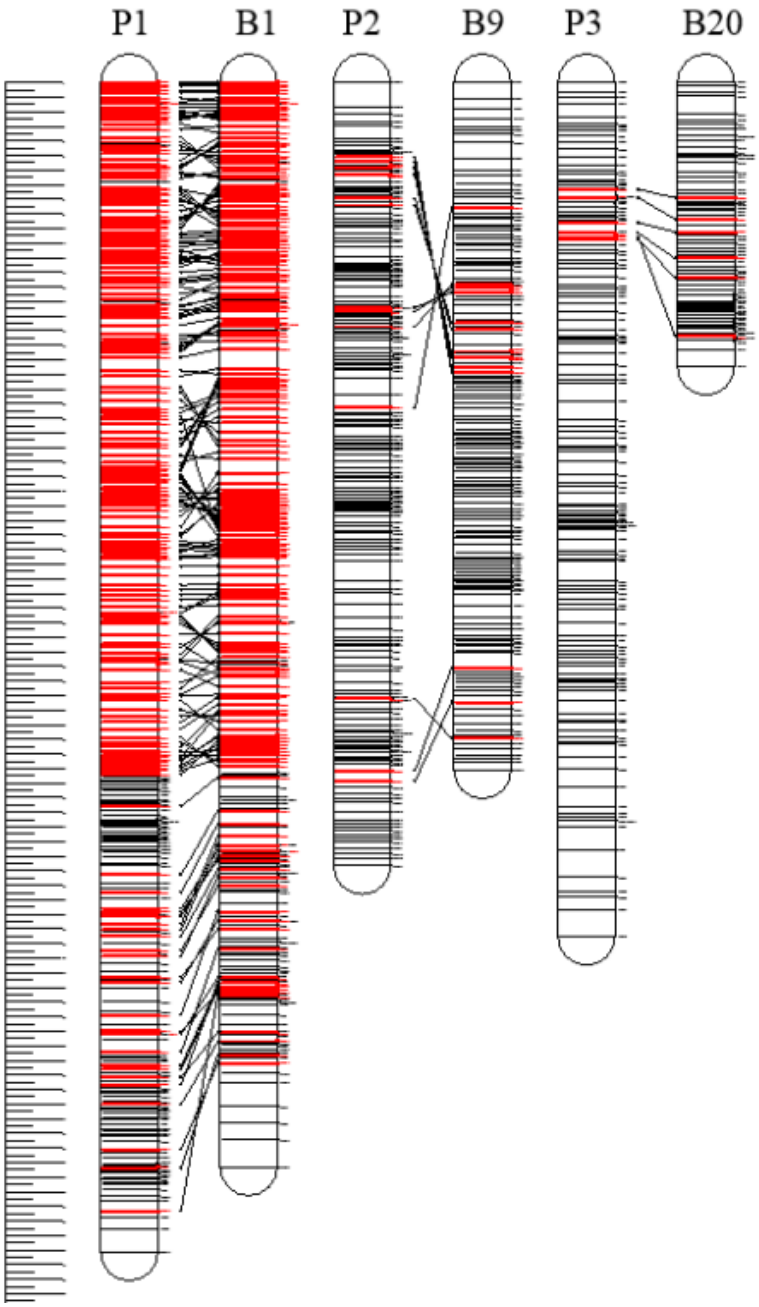


Fig. 5.1. Preliminary single nucleotide polymorphism based genetic linkage map for *S. meyeri* 'Palabin' (P) and *S. pubescens* 'Penda' Bloomerang® (B). Scalebar in centi-Morgans (cM) with large tick marks representing 10 cM increments. Lines represent marker postions on each linkage group. Markers on female map (P) composed of lm x ll (black) and hk x hk (red) markers. Markers on male map (B) composed of nn x np (black) and hk x hk (red) markers. Lines connecting male and female linkage groups are based on shared hk x hk markers.

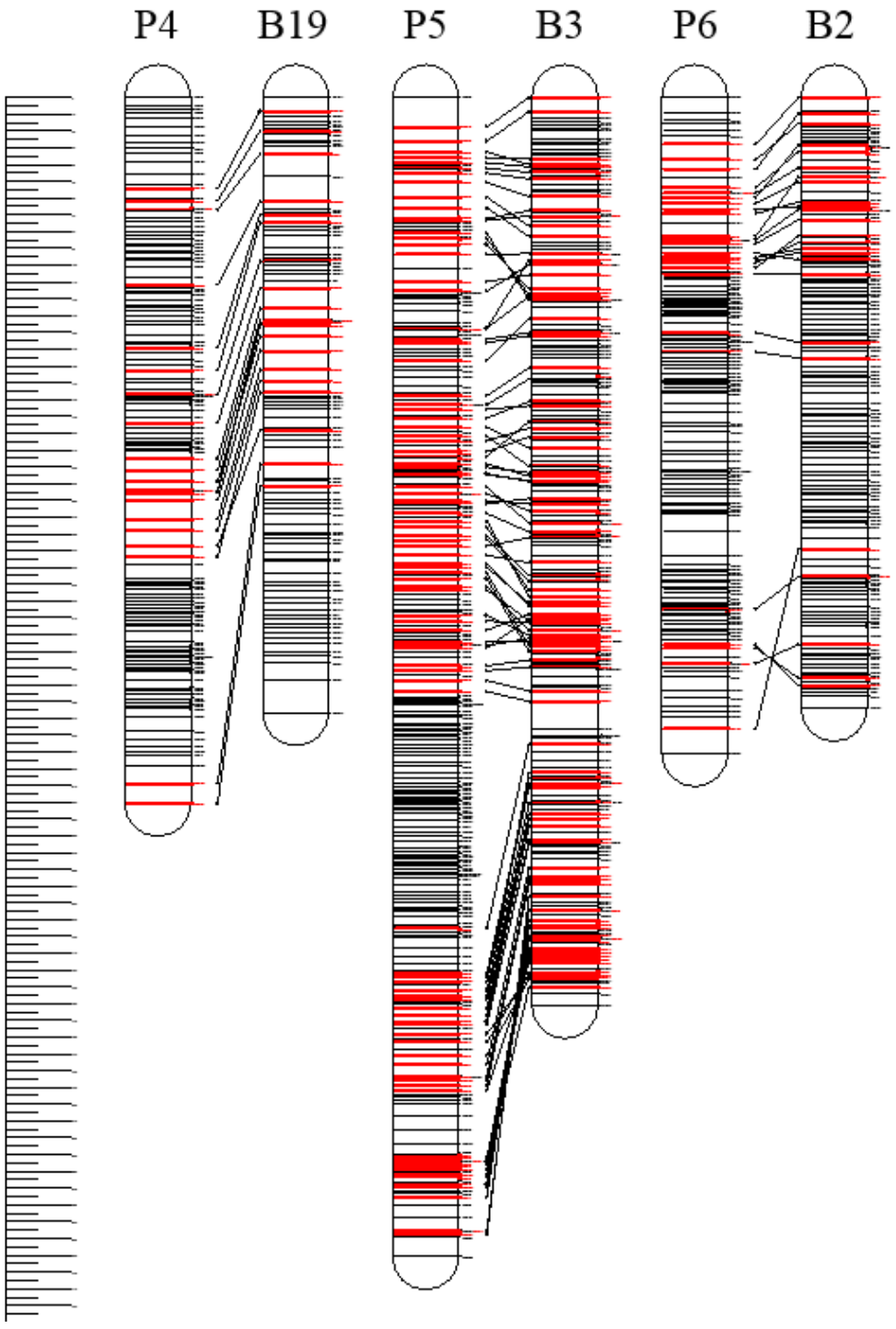
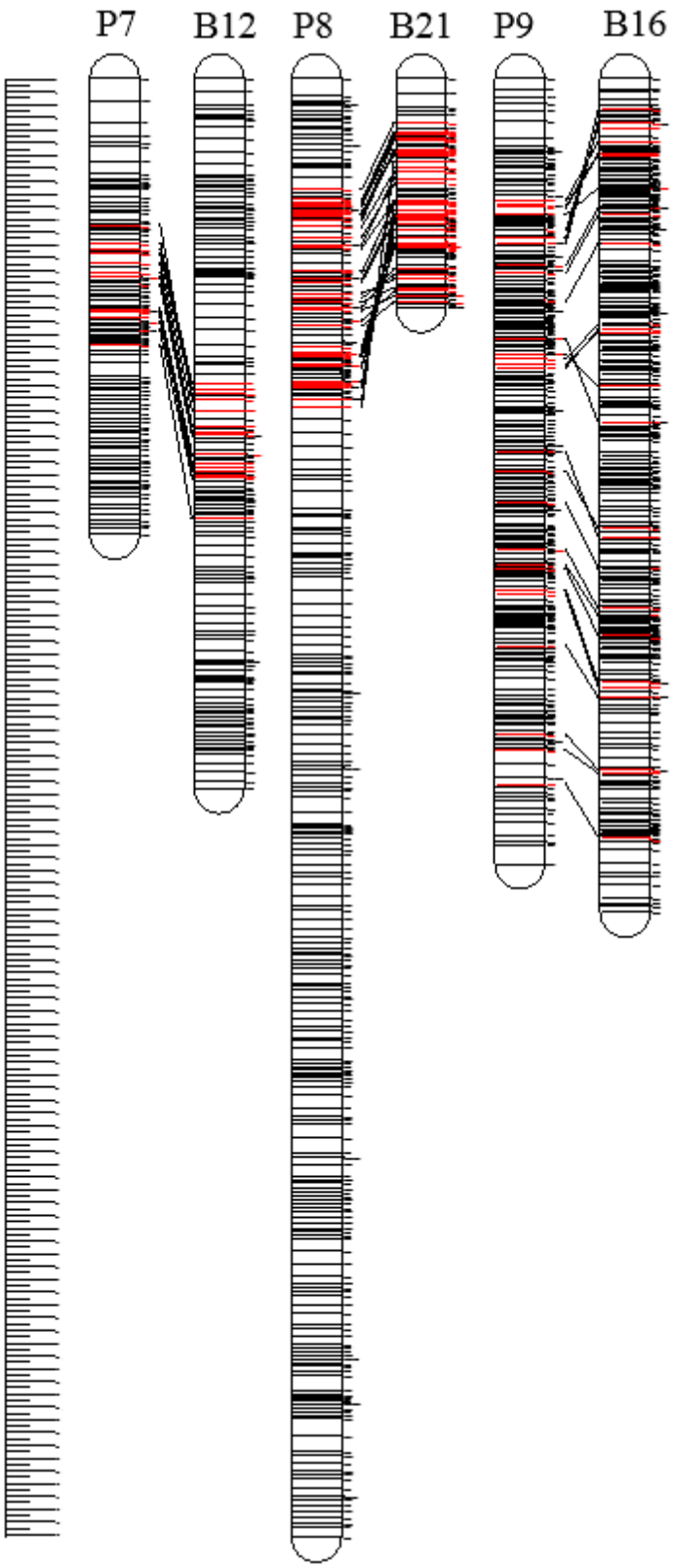


Fig. 5.1 (continued).



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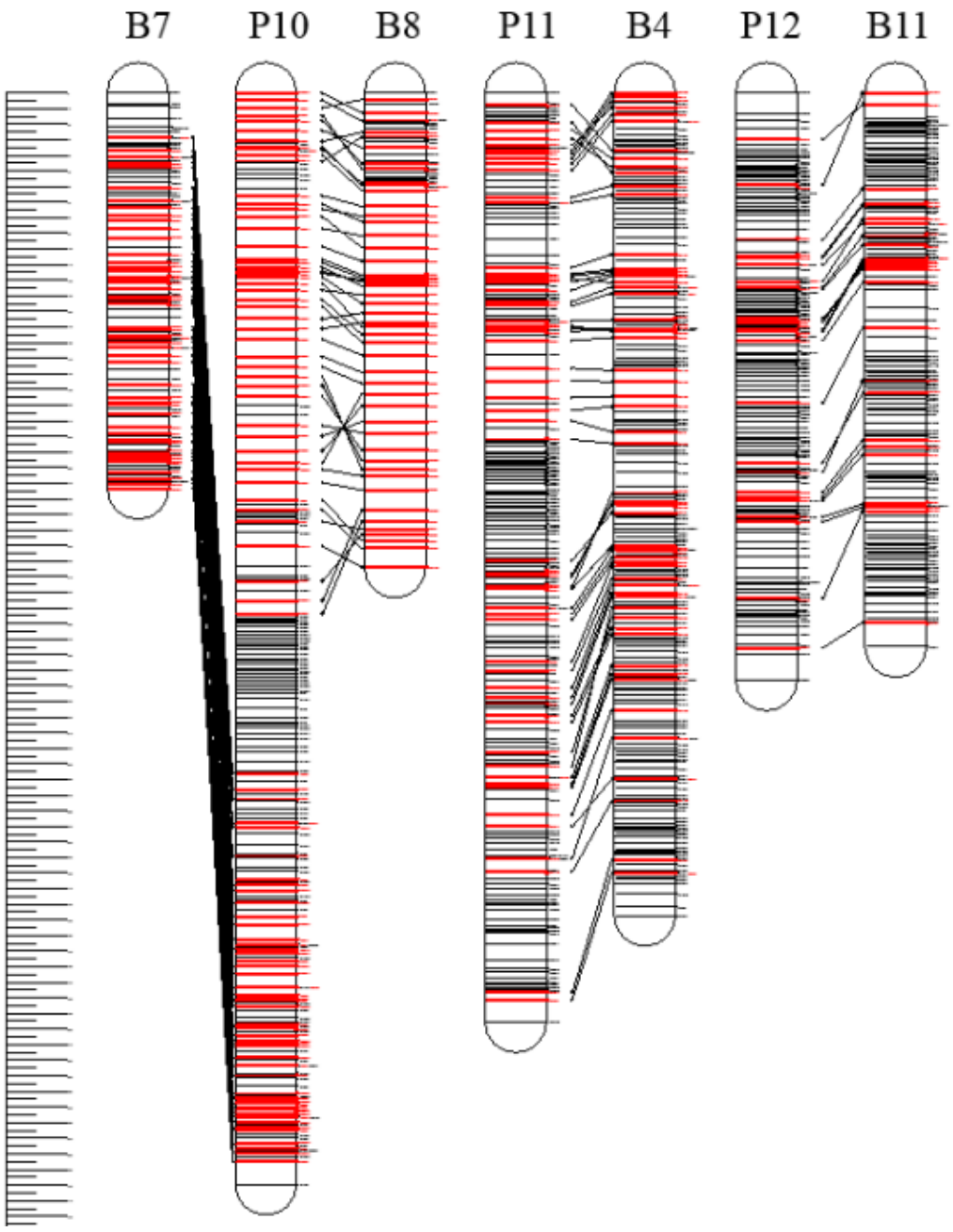
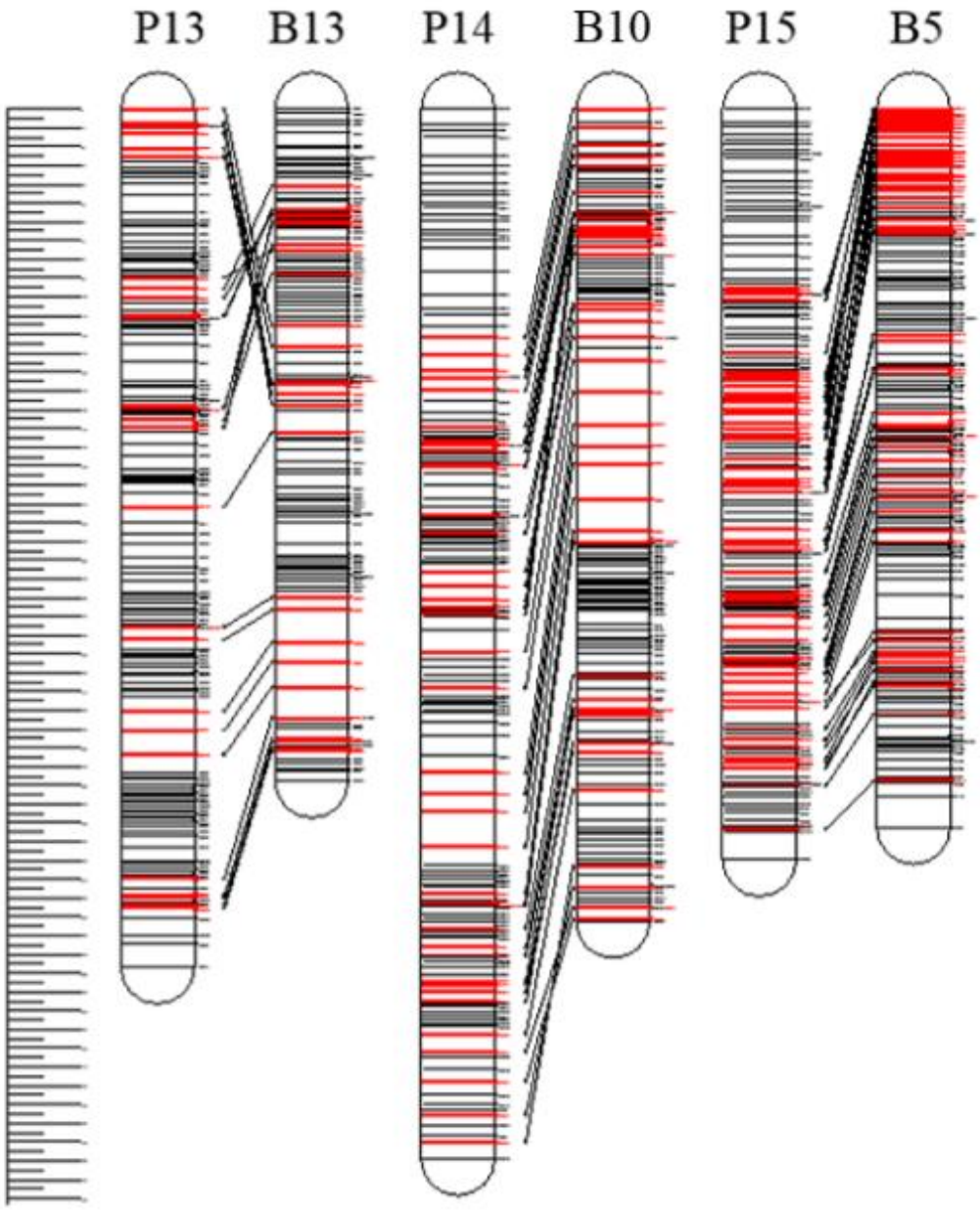


Fig 5.1 (continued).



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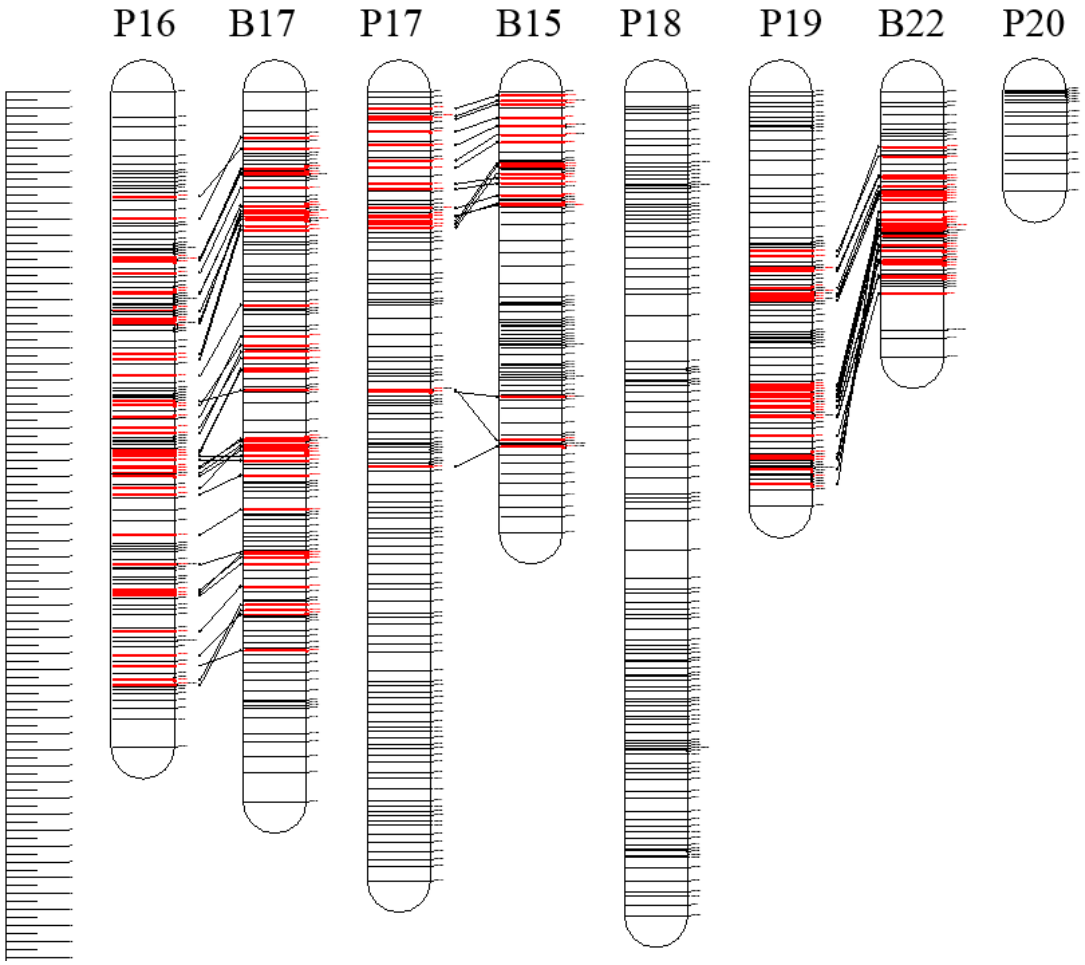

Fig. 5.1 (continued).

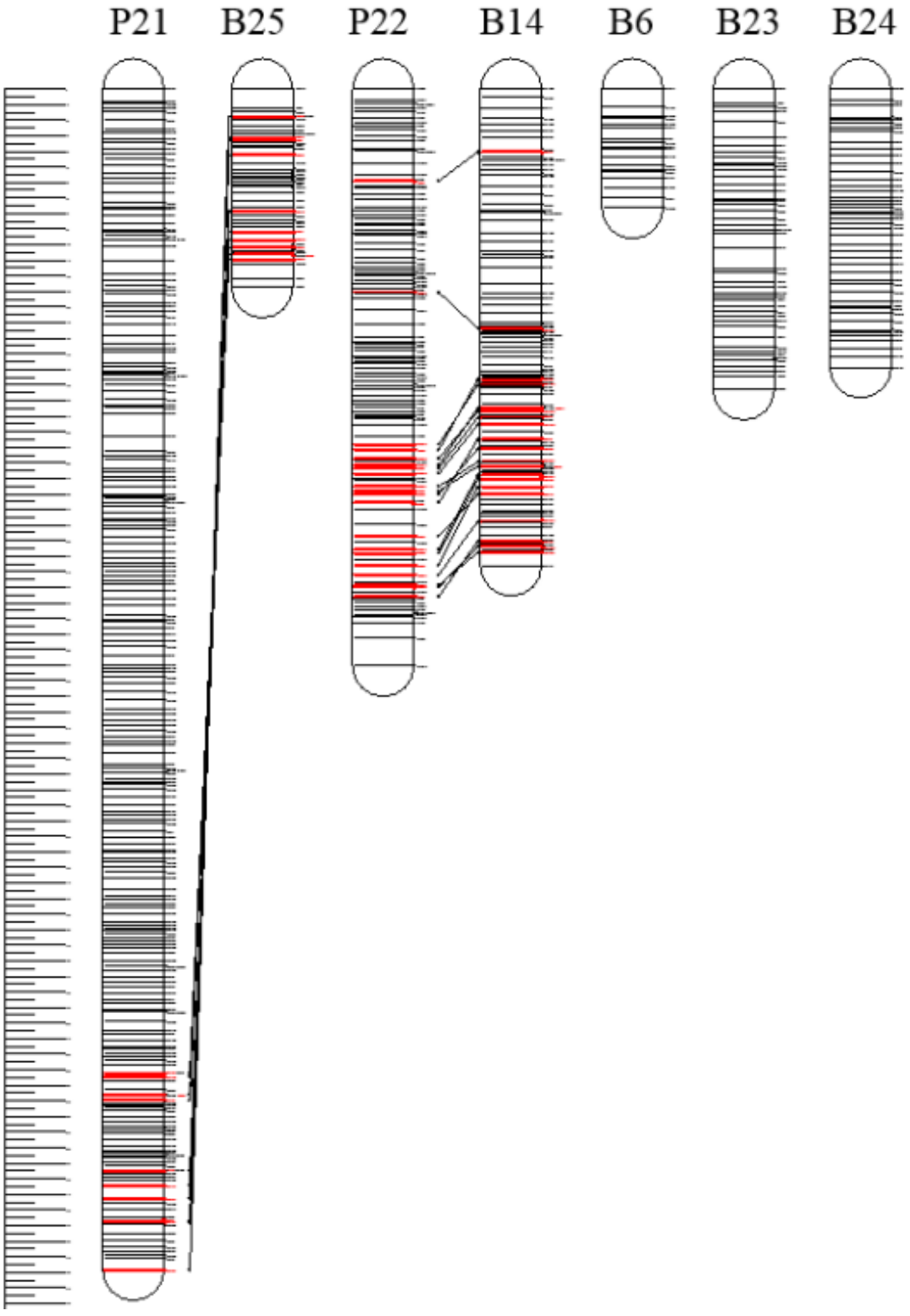

Fig. 5.1 (continued).

CHAPTER 6: VARIATION IN GENOME SIZE, PLOIDY, STOMATA, AND rDNA SIGNALS IN ALTHEA (*Hibiscus syriacus* L.).

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Additional index words: flow cytometry, cytology, holoploid genome size, spindle-fiber inhibitors, ploidy series, stomata, fluorescent in situ hybridization, 5S rDNA, 45S rDNA

Abstract. Althea (*Hibiscus syriacus*) is an ornamental shrub prized for its winter hardiness and large colorful summer flowers. Althea are primarily tetraploids ($2n = 4x = 80$) with higher level polyploids reported from experiments with spindle-fiber inhibitors. Previous studies report anatomical variation among althea polyploids, including changes in stomata size. The purpose of this study was four-fold. The first was to identify genome size and ploidy variation in althea cultivars via flow cytometry and chromosome counts. The second was to create a ploidy series consisting of 4*x*, 5*x*, 6*x*, and 8*x* cytotypes using a combination of interploid hybridization and autopolyploid induction via colchicine and oryzalin. The third was to investigate the ploidy series for variation in stomatal guard cell lengths, stomatal density, and copy number of fluorescent rDNA signals. The fourth was to investigate segregation patterns in rDNA signals in a subset of putative pentaploid seedlings. Flow cytometry revealed the majority of cultivars to be tetraploid with holoploid 2C genome sizes ranging from 4.55 ± 0.02 pg to 4.78 ± 0.06 pg. Five taxa (‘Aphrodite’, ‘Pink Giant’, ‘Minerva’, Azurri Satin®, and Raspberry Smoothie™) were hexaploids

with significantly larger genome sizes ranging from 6.68 ± 0.13 pg to 7.05 ± 0.18 pg. A single taxon, Peppermint Smoothie™, was a cytochimera with both tetraploid cells (4.61 ± 0.06 pg) and octaploid cells (8.98 ± 0.13 pg). To create pentaploids, 935 pollinations were performed in 43 reciprocal combinations among hexaploid 'Pink Giant' and tetraploid cultivars. Viable seedlings were recovered from 16 combinations and pentaploids were confirmed by flow cytometry. Agar solutions containing 0.2% colchicine and 125 μM oryzalin were both successful at creating octaploids by treating seedling meristems. Four taxa were selected to represent the four cytotypes in the ploidy series: tetraploid [Bali™ (4.61 ± 0.00 pg)], pentaploid ['Pink Giant' x Bali™ (5.55 ± 0.02 pg)], hexaploid ['Pink Giant' (6.97 ± 0.07 pg)], and octaploid [oryzalin-treated, open-pollinated seedling from Bali™ (8.88 ± 0.01 pg)]. Measurements of stomatal guard cells revealed significant differences in average guard cell lengths among the four cytotypes: 4*x* (27.35 ± 0.04 μm), 5*x* (30.35 ± 1.28 μm), 6*x* (35.59 ± 0.63 μm), and 8*x* (8.88 ± 0.01 μm). Stomatal guard cell lengths proved to be a reliable measure of ploidy in *H. syriacus*. Measurements of stomatal density revealed a precipitous decline in average density from the 4*x* cytotype (398.22 ± 15.43 stomata·mm^{-2}) to 5*x* cytotype (194.06 ± 38.69 stomata·mm^{-2}), and no significant difference was found among 5*x*, 6*x*, and 8*x* cytotypes. Fluorescent in situ hybridization revealed an increase in 5S and 45S rDNA signals that scaled with ploidy: 4*x* (two 5S + four 45S), 6*x* (three 5S + six 45S), and 8*x* (four 5S + eight 45S). However, pentaploid (5x) seedlings exhibited random segregation of rDNA signals between the 4*x* and 6*x* cytotypes including all six possible combinations [two 5S, three 5S] x [four 45S, five 45S, six 45S]. The rDNA loci

confirmed ploidy levels in each cytotype of our ploidy series, and random segregation of rDNA loci provides evidence of random chromosome segregation in interploid hybrids of althea.

Introduction

Hibiscus is a genus in the mallow family (Malvaceae) which represents approximately 250 species of mostly tropical and sub-tropical trees, shrubs and herbs divided into ten sections (Van Laere, 2008; Fryxell, 1988). Within this vast genus, few species extend their natural range into temperate climates; temperate species include *H. paramutabilis*, *H. sinosyriacus*, and *H. syriacus* (Bates, 1965). Rose-of-sharon or althea (*H. syriacus*) has been a staple ornamental shrub in American gardens prized for its winter hardiness, range of flower colors, and unique flower phenotypes including single-flowered, double-flowered, and semi-double (anemone) types (Contreras and Lattier, 2014). In 2014, total nationwide sales of *Hibiscus* topped 4.4 million units, generating over $30 million in revenue (USDA, 2016). Breeders have noted the potential for improvement in *H. syriacus* due to their range of flower color and form and their short generation time from seed to flower (Dirr, 2009).

The basic chromosome number of *H. syriacus* has been reported as $x = 20$ with most being tetraploid, $2n = 4x = 80$ (Skovsted, 1941). However, confusion persists in the literature as some reports claim *H. syriacus* to exist primarily as a diploid. Van Laere et al. (2007) addressed this discrepancy confirming that the term “diploid” is often used instead of the correct term “tetraploid” which has led to

confusion in classifying hexaploid cultivars (so-called triploids) and octaploid cultivars (so-called tetraploids). A recent draft genome analysis and annotation revealed high copy numbers in numerous genes compared to closely related diploid species, confirming that *H. syriacus* is a tetraploid (Kim et al., 2017). Therefore, to avoid confusion, all ploidy level reports have been adjusted to a base tetraploid cytotype according to Skovsted (1941) and indicated with an asterisk [e.g. "*H. syriacus* 'Diana' is reported as a triploid $2n = 3x = 120$ (Shim et al., 2003)" becomes "*H. syriacus* 'Diana' is reported as a hexaploid $2n = 6x^* = 120$ (Shim et al., 2003)."] (Table 6.1). Although the majority of taxa investigated are tetraploid, higher level polyploids have been reported including hexaploids ('Aphrodite', 'Diana', 'Helene', 'Minerva', 'Melrose', 'Pink Giant', and 'Shimsan') and octaploids ('Purple CV', 'Purple CV_2', 'Red Heart CV', 'Sp_1', Sp_2', and 'Sd_1') (Table 6.1).

Spindle fiber inhibitors (SFIs) including colchicine and oryzalin have been used successfully to create higher level polyploids in *Hibiscus*. Colchicine, a chemical produced by the autumn crocus (*Colchicum autumnale*), has been used since the 1930s to promote polyploidy in plants (Blakeslee and Avery, 1937). However, its higher affinity for animal microtubules than plant microtubules makes it dangerous to handle while requiring high concentration to be effective in plant cells (Dhooge et al., 2010; Morejohn et al., 1984). Oryzalin is the active ingredient in the dinitroaniline herbicide Surflan (United Phosphorous, Trenton, NJ). Oryzalin has a high affinity for plant tubulin dimers allowing it to be used at low concentrations, reducing its toxicity to humans while reducing incidences of abnormal growth and mutations in induced

polyploids (Bajer and Molebajar, 1986; Dhooge et al., 2010; Hugdahl and Morejohn, 1993; Morejohn et al., 1987).

Contreras et al. (2009) treated meristems of germinating seedlings with an agar solution containing 100 μM to 150 μM oryzalin to induce polyploidy in *H. acetosella*. However, seeds soaked in a colchicine solution at concentrations of 0.2% to 0.5% failed to produce polyploids (Contreras et al., 2009). Li and Ruter (2017) soaked seedlings in colchicine (0.025% to 0.1%) and oryzalin (100 to 150 μM) solutions to induce autopolyploids in *H. moscheutos*. Hexaploids 'Aphrodite', 'Diana', 'Minerva' and 'Helene' of *H. syriacus* were created at the US National Arboretum (USNA) by crossing improved selections with colchicine-treated seedlings of 'William R. Smith'. Ploidy of the hexaploids 'Melrose' and 'Pink Giant' remained unreported prior to a flow cytometry survey by Van Huylenbroeck et al. (2000). Hexaploid 'Shimsan' as well as the octaploid taxa 'Purple CV, 'Purple CV_2', and 'Red Heart CV' were developed at Sung Kyun Kwan University in Korea (Shim et al., 1993). Lee and Kim (1976) used 0.2% to 0.5% colchicine solutions to create octaploid *H. syriacus* individuals 'Sp_1', 'Sp_2', and 'Sd_1' at the Institute of Forest Genetics in Korea. Van Laere et al. (2006) created hexaploid lines of blue-flowered *H. syriacus*. A colchicine solution (0.2%) was applied by agar droplet, immersion, and filter paper to create octaploid seedlings of 'Blue Bird' and 'Woodbridge', of which the droplet method proved most efficient (Van Laere et al., 2006). Octaploid seedlings were then crossed with tetraploid cultivars and a single taxon was selected, Azurri Satin® (Van Laere et al., 2006).

Ploidy levels and chromosome numbers in cultivars and induced polyploids of *Hibiscus* have been confirmed in previous studies using combinations of flow cytometry and root squashes (Contreras et al., 2009; Shim et al., 1993; Van Huylenbroeck et al., 2000; Van Laere et al., 2006, 2009). In addition to root squashes, anatomical differences among putative *Hibiscus* polyploids have been investigated. Observations of morphological characters have consistently shown "gigas" effects among polyploids, with polyploids having larger flowers, an increased flowering duration, and a reduction in seed production (Egolf, 1971; 1981b; Van Huylenbroeck et al., 2000). In *H. acetocella*, Contreras et al. (2009) recorded differences in plant height, leaf size, internode length, canopy volume, pollen diameter and guard cell length among polyploids. In *H. syriacus*, Lee and Kim (1976) recorded differences across ploidy for leaf thickness, length of guard cells, diameter of pollen grains, lengths of wood fibers, and widths of wood fibers. Shim et al. (1993) reported differences in leaf width, leaf length, flower size, eyespot size, pollen spine number, and pollen exine apertures associated with differences in ploidy in *H. syriacus*. Skovsted (1941) attempted to separate putative polyploids based on seed size in several species of *Hibiscus*. Van Laere et al. (2009) investigated pollen diameter differences and triad formation among polyploids of *H. syriacus*.

Not all morphological characters are equal at determining genome size and ploidy changes in plants. One of the more reliable characters is stomatal guard cell size and density. Genome size has been shown to have a positive correlation with stomata size and a negative correlation with stomata density across a wide range of angiosperms (Beaulieu et al., 2008). Stomata have proven useful anatomical

characters to differentiate ploidy levels in woody plant species such as hawthorns (McGoey et al. 2014), roses (Joly and Bruneau, 2007), and citrus (Padoan et al., 2013). In fact, stomatal guard cells have provided a robust enough character that they have been useful for predicting ploidy in dried herbarium specimens in taxa such as *Buddleja* (Chen et al., 2009), *Salix* (Buechler, 2000), and *Phragmites* (Saltonstall et al., 2007), and even fossil leaves in *Salix* (Buechler, 2000). Stomata measurements and anatomical descriptions have been reported for many species of *Hibiscus* (Contreras et al., 2009; Essiett and Iwok, 2014; Li and Ruter, 2017; Zhuang and Song 2005), yet only one study has reported an association of ploidy variation on stomata in *H. syriacus* (Lee and Kim, 1976). Guard cell lengths of tetraploid controls ranged from 28 to 29 μm while those of colchicine-induced octaploids ('Sp_1', 'Sp_2', and 'Sd_1') ranged from 32 to 42 μm.

Confirming ploidy level in woody plants can be performed with flow cytometry and traditional cytology, such as root squashes. Root squashes can be difficult and tedious as many woody plants possess small, friable roots with numerous, small chromosomes (Lattier et al., 2013; Ochatt, 2008). Fluorescent labelling of ribosomal DNA (rDNA) could be used to confirm ploidy level in a ploidy series with numerous, small chromosomes, as well as provide a tool for investigating chromosome segregation through copy number variation in rDNA signals of interploid hybrids. However, this may not be a viable alternative due to time and difficulty of the technique, or if variation in rDNA signals exists within ploidy levels.

Ribosomal DNA has been used to study the origin and evolution of plant genomes in ancient allopolyploids (Volkov et al., 2017) as well as artificial

autopolyploids (Gomes et al., 2014) and interploid hybrids (Wang et al., 2015a). Tandem repeated rDNA units are highly conserved throughout all plants and are often combined with more variable, rapidly evolving intergeneric spacer regions (IGS) (Volkov et al., 2017). Frequently used loci include the 5S rDNA, which includes the 5S rRNA repeated units plus IGS, and the 45S rDNA (also known as nucleolar organizer regions, NORs), which includes the 18S, 5.8S, and 25S rRNA repeated units, internal transcribed sequences (ITS1 and ITS2) and IGS spacer regions (Ribeiro et al., 2008; Volkov et al., 2017). The 45S and 5S rDNA loci are usually located at different sites on different chromosomes, and their transcription is carried out by different RNA polymerases (Srivastava and Schlessinger, 1991). These markers have become widely used in fluorescent in situ hybridization (FISH), a technique that fluorescently labels rDNA and allows for comparison of copy number and location. FISH has proven an efficient technique for cytological studies in woody angiosperms where karyotyping is limited due to the small size of chromosomes as demonstrated in birch (Anamthawat-Jónsson, 2003) and poplar (Prado et al., 1996).

The objectives of this study were 1) to identify genome size and ploidy variation in cultivars of *H. syriacus*, 2) to create a ploidy series consisting of 4*x*, 5*x*, 6*x*, and 8*x* cytotypes using a combination of interploid hybridization and autopolyploid induction via colchicine and oryzalin, 3) to investigate the ploidy series for variation in stomatal guard cell length, stomatal density, and copy number of fluorescent rDNA signals, and 4) to investigate segregation patterns in rDNA signals in a subset of putative pentaploid seedlings.

Methods and Materials

Plant material. Vegetative cuttings and container plants of *H. syriacus* were collected from nurseries, gardens, and arboreta to represent a cross section of available cultivars in the nursery trade (Table 6.2). Plants were maintained in containers at the Lewis Brown Farm at Oregon State University in Corvallis, OR. Original cultivar and trademark names were maintained from each source (Table 6.2); however, usually one name becomes common in the nursery trade as the market name. For simplicity, only market names (cultivar or trademark) will be used hereafter.

Flow cytometry. Holoploid (2C) relative genome sizes were recorded for each accession of *H. syriacus*. For each plant, three recently expanded leaves were randomly collected to represent three samples of nuclei for each accession. A single leaf was sampled from additional clones of each taxon. If genome size variability was found among additional "clones," then three leaves were screened and additional genome sizes were reported for those accessions, as in *H. syriacus* 'Aphrodite' (Table 6.3). For each leaf sample, an internal standard of known genome size was included (*Solanum lycopersicum* 'Stupicke'; 2C = 1.96 pg). Combined leaf tissues (1-2 cm^2) representing a sample plus internal standard were co-chopped in 400 µL of a buffer solution (Cystain Ultraviolet Precise P Nuclei Extraction Buffer; Sysmex, Görlitz, Germany). The resulting solution was poured through a 30-µm gauze filter (Partec Celltrics, Münster, Germany) into a 3.5-mL plastic tube (Sarstedt Ag & Co.; Nümbrecht, Germany) followed by 1.6 µL of fluorochrome stain (DAPI; 4',6-diamidino-2-phenylindole) (Cystain ultraviolet Precise P Staining Buffer; Partec).

The nuclei suspension was analyzed using a flow cytometer (CyFlow Ploidy Analyzer; Partec) with a minimum of 3000 nuclei analyzed per sample at a coefficient of variation (CV) for each histogram less than ten. References to genome size and ploidy follow the terminology proposed by Greilhuber et al. (2005). Holoploid genome size was calculated as:

$$2C = \text{DNA content of standard} \times \frac{\text{mean fluorescence value of sample}}{\text{mean fluorescence value of standard}} .$$

Fluorescence histogram figures were created using open source software from Purdue University Cytometry Laboratories (PUCL, 2014).

Cytology. To calibrate the genome sizes from flow cytometry with ploidy levels, a root squash was performed on a tetraploid cytotype following the protocol of Lattier et al. (2017). Rooted cuttings of 'Diana' were used for root tip collection. Root tips were collected before 1000 HR following a sunny day and treated in 1.5-mL microcentrifuge tubes containing a pre-fixative solution of 2 mM 8-hydroxyquinoline + 0.24 mM cycloheximide. Root tips were maintained at room temperature for 2.5 h before a cold treatment at 4 °C for another 2.5 h. Root tips were rinsed in filter-sterilized water and fixed overnight in Carnoy's solution (6 parts 95% ethanol: 3 parts chloroform: 1 part glacial acetic acid; by volume).

The following day, root tips were rinsed with sterile water and stored in 70% ethanol in a refrigerator at 4 °C. Enzyme digestions were performed for 2 to 3 h. Enzyme digestion solution consisted of 0.5% cellulase (from *Trichoderma reesei*; Sigma-Aldrich, St. Louis, MO), 0.5% cytohelicase (from *Helix pomatia*; Sigma-Aldrich) and 0.5% pectolyase (from *Aspergillus japonicus*; Sigma-Aldrich) in a sodium citrate buffer at pH = 4.5. Metaphase chromosomes were screened at

magnification ×200 using a compound light microscope (Axio Imager A1; Zeiss Microscopy, Oberkochen, Germany). Fifteen highly resolved cells were imaged under oil immersion at a magnification of ×1000. Chromosomes were imaged at different focal distances and focus-stacked using the Auto Blend utility in Photoshop CC 2015.5.1 (Adobe Systems; San Jose, CA). For higher level polyploids (5*x*, 6*x*, and 8*x*), ploidy levels were confirmed using root squashes combined with rDNA signal variation from FISH analysis (details below).

Ploidy series. From 2013 to 2015, interploid hybridization and induced autopolyploidy were used to create a ploidy series (4*x*, 5*x*, 6*x*, and 8*x*). Interploid hybridizations were performed between a hexaploid taxon identified through flow cytometry, 'Pink Giant', and a suite of tetraploid cultivars. A total of 935 pollinations were performed with 'Pink Giant'. When using 'Pink Giant' as a seed parent, 379 pollinations were attempted representing 19 different combinations. When using 'Pink Giant' as a pollen parent, 556 pollinations were attempted representing 24 different combinations. Controlled pollinations were performed in summer in a glasshouse kept free of pollinators with day/night temperatures set at 25/20 °C and a 16-h photoperiod. Dried capsules were collected prior to dehiscence in fall. Non-stratified seed from each cross were directly sown into 1.3-L containers filled with growing medium (Metro-Mix; Sun Gro Horticulture, Agawam, MA) and seeds were evenly spaced at 30 or fewer seeds per pot. A subset of seedlings from each successful cross were screened using flow cytometry according to the methods described above. A vigorous selection with an intermediate, pentaploid genome was selected for the ploidy series, H2013-078-01 ('Pink Giant' x Bali™).

To create octaploid cytotypes, an autopolyploid induction experiment was designed in 2014 to treat germinating seedling meristems with SFIs at different concentrations and durations. Open-pollinated (OP) seeds were collected from 'Aphrodite' and Bali™ and directly sown into 1.3-L containers filled with growing medium (Metro-Mix) (Fig. 6.1A). Replicates of five pots with subsamples of 15 seeds per pot were used for each treatment. Pots were grown under cool-white fluorescent lights at 90 $mmol \cdot m^{-2} \cdot s^{-1}$ at 22 to 25 °C with a 16-h photoperiod.

As seedlings randomly germinated over several months, autopolyploid induction treatments were randomly applied to the containers. Two genotypes ('Aphrodite' and Bali™) were treated with two SFIs [oryzalin (Surflan AS; United Phosphorous) and colchicine (Sigma-Aldrich)] in five treatment combinations: 125 μM oryzalin for five days, 125 μM oryzalin for ten days, 125 μM oryzalin for twenty days, and 0.2% colchicine for ten days. Colchicine at 0.2% was effective for *H. syriacus* (Van Laere et al., 2006), however use of oryzalin has not been reported for *H. syriacus*. In addition, a negative control was included (SFI-free agar droplet for ten days). Droplets were applied daily as seedling cotyledons opened, revealing the developing meristem, and each day's treatment was marked with a colored toothpick (red/orange/yellow for one day, blue for five days, green for ten days) (Fig. 6.1A). Containers were covered with clear humidity domes to maintain the treatment droplets (Fig. 6.1B). Containers were held in non-draining plastic trays and seedlings were bottom watered as needed to keep the surface of the plants free of water.

To improve cellular penetration, 1% (v/v) dimethyl sulfoxide (DMSO) was added to the colchicine treatment. To congeal all treatments, including the control,

and maintain droplets on the meristems over the course of each day, 0.55% agar (w/v) (Sigma-Aldrich) was added to each treatment. Treatment solutions were stored in 125-mL Erlenmeyer flasks. Before each application, treatments were microwaved in 10 to 15 second intervals until the agar completely liquefied and began to bubble. Flasks were moved to a fume hood where they were allowed to cool, but kept warm on a hot plate and kept well-mixed using a magnetic stir bar. After preparing all treatments, flasks were sealed and moved to a water bath at 38 °C until the daily treatments were complete.

After all treatments were complete, 13 germinated seedlings from each container were transplanted into individual 0.95-L containers and randomized on a glasshouse bench under the culture conditions described above (Fig. 6.1C). As plants grew, single and two-node cuttings were taken from developing plants due to necrosis at the treatment site for many seedlings (Fig. 6.1D). Several developing roots were collected from a subset of treatment plants and a single sample was analyzed for each via flow cytometry to identify putative octaploid. A vigorous selection (OP2014-19) with an octaploid genome size resulting from the 125 μM oryzalin – five day treatment was selected for the ploidy series. The following year, young expanded leaves were analyzed via flow cytometry to confirm the ploidy level of this selection. The final ploidy series was comprised of four accessions: Bali™ (4*x*), H2013-078-01 'Pink Giant' x Bali™ (5*x*), 'Pink Giant' (6*x*), and OP2014-19 oryzalin-treated OP Bali™ seedling (8*x*).

Stomata measurements. In summer 2016, four clonal plants representing the ploidy series were growing in the same environment at the Lewis Brown Farm,

Corvallis, OR. Three mature leaves for each plant were randomly selected from different branches. Similar sized leaves were selected (Fig. 6.2A) approximately three to four nodes basipetal to the developing meristem. For each leaf, an area between the midrib and first primary vein on the abaxial leaf surface was treated with a thin coat of clear, nitro-cellulose fingernail polish (Fig. 6.2B). Strips of clear packing tape were applied to the nail polish. After allowing the nail polish to dry (five to ten minutes), the tape strips were carefully removed with forceps (Fig. 6.2C). The resulting cuticle peels containing relief impressions of stomata were mounted to microscope slides (Fig. 6.2D). Each slide representing a random leaf sample was treated as a replicate for further analysis. Each slide was viewed with a compound light microscope (Axio Imager A1; Zeiss) at a magnification of ×200.

Images were randomly captured across the microscope slides (AxioCam 105 Color; Zeiss) and processed using image analysis software (AxioVision; Zeiss). Five to ten images totaling 97 to 289 stomata per slide were used to measure stomatal guard cell length for each cytotype. Total stomata measured (2281) for each cytotype were: 799 (tetraploid), 433 (pentaploid), 524 (hexaploid), and 525 (octaploid). All stomata were measured in each image using the line measurement tool (AxioVision; Zeiss). Stomata per slide were treated as subsamples and stomatal guard cell lengths were averaged for each slide. Average guard cell lengths per slide were averaged for a total of three reps per cytotype. For stomatal density, 50 randomly captured images per slide were analyzed for a total of 150 images per cytotype. Total stomata counted (21,015) for each cytotype were: 9029 (tetraploid), 4400 (pentaploid), 4095 (hexaploid), and 3491 (octaploid). The number of stomata was counted in each

image and an average stomata number per slide was calculated. Average stomata counts for each slide were averaged for a total of three reps per cytotype. To report stomatal density (stomata·mm^{-2}), each stomata count was multiplied by 6.62 to scale up from the frame of view at a magnification of ×200 (449.2 μm × 336.5 μm) according to the following formula:

$$\text{Stomatal density (stomata}\cdot\text{mm}^{-2}) = \text{stomata count} \times \frac{1}{(449.2\ \mu\text{m} \times 336.5\ \mu\text{m}) \times 1e^{-6}}.$$

After guard cell lengths and stomatal densities were calculated, stomatal index was calculated according to Li et al. (1996):

$$\text{Stomatal index} = \text{stomata length } (\mu\text{m}) \times \text{stomata density (stomata}\cdot\text{mm}^{-2}).$$

Photomicrographs of cuticle peels from each cytotype were produced from layered images composed of multiple focal distances viewed at a magnification of ×630. Images were layered using the Auto Blend feature in Photoshop (Adobe Systems). Average stomata estimates (as well as ploidy levels) were subjected to analysis of variance (ANOVA) and means were separated using Fisher's least significant difference ($\alpha < 0.05$) (SAS Studio; Cary, NC).

FISH. Synthesis of probes for FISH were carried out according to Chang et al. (2009). A plasmid DNA construct from wheat, p*TA794* (Gerlach and Dyer, 1980), containing the 5S rDNA repeat (410 bp) was labelled with digoxigenin (DIG-11-

dUTP) by nick translation (Roche Diagnostics; Mannheim, Germany). The digoxigenated probe was represented by a red fluorescent signal, detected using Texas red (anti-digoxigenin-rhodamine Fab fragments) (14877500; Roche Diagnostics). Another plasmid DNA from wheat, p*TA71* (Gerlach and Bedbrook, 1979), containing ~9kb of coding sequences from the 45S rRNA gene (18S-5.8S-26S) was labeled with biotin (Biotin-16-dUTP) by nick translation (Roche Diagnostics). The biotinylated probe was represented by a green fluorescent signal, detected using fluorescein anti-biotin (SP-3040; Vector Laboratories, Burlingame, CA). Counterstaining of chromosomes was performed with DAPI suspended in a mounting medium at 1.5 $\mu g \cdot mL^{-1}$ (Vectashield; Vector Laboratories).

Roots tips for FISH were collected from softwood cuttings of *H. syriacus*. Softwood cuttings were rooted from plants including tetraploid Bali™, hexaploid 'Pink Giant', octaploid OP2014-19, and a random sample of putative pentaploid seedlings from interploid crosses. Root tip pre-fixative, fixative and enzyme digestion steps follow protocol listed above (*Cytology*). After digestion, the enzyme solution was wicked away using low-lint tissue (VWR International; Radnor, PA) and roots were squashed according to the protocol by Chang et al. (2009). Two drops of modified Farmer's solution (3 parts 95% methanol: 1 part glacial acetic acid by volume) were added to each slide and macerated root tip cells were dispersed by lightly tapping with a metal spatula. Four drops of modified Farmer's solution were added to the corners of the slide before passing the slide over an alcohol lamp. As the flame dissipated, slides were allowed to dry prior to staining.

Dried slides were treated for 15 min in diluted Giemsa stain (Sigma-Aldrich), rinsed in water, and allowed to dry. Stained slides were screened for condensed chromosomes at a magnification of ×200 on a light microscope (Axio Imager A1; Zeiss). High quality slides with condensed chromosomes were selected for FISH. Giemsa stain was removed by incubating slides (Coplin jars) into -20 °C chilled Farmer's solution (3 parts 95% ethanol: 1 part glacial acetic acid; by volume) for 5 min followed by 5-min incubations in 75%, 95% and 100% ethanol. After air-drying, slides were placed in an oven at 60 °C for at least 30 min.

Preparation for FISH analysis was carried out according to previous methods (Chen et al., 2015; Chung et al., 2008) modified for *H. syriacus*. Slides were removed from oven and incubated in 10 mM HCl for 5 min. Next, a solution containing 0.1% pepsin in 10 mM HCl (w/v) was applied to each slide (150 μL per slide) and covered with a plastic cover slip. Pepsin treatments were carried out at 37 °C for 1 hr. After removing the plastic cover slip, slides were incubated in 10 mM HCl for 5 min followed by incubation in 2× saline-sodium citrate buffer (SSC) for 5 min. Next, a solution containing 70% formamide and 2× SSC (v/v) was added to each slide (30 μL per slide) before covering with a plastic cover slip. Slides were incubated at 80 °C for 50 to 60 seconds to denature the DNA. Then, plastic cover slips were removed and slides were incubated in iced 75% ethanol, followed by room temperature 95% and 100% ethanol for 5 min each and allowed to dry. As slides dried, a probe mix (20 μL per slide) was constructed, incubated at 90 °C for 10 min, and put on ice for 5 min. The probe mix was composed of salmon sperm DNA (4 μL), 5S rDNA probe (4 μL), and 45 rDNA probe (4 μL) which were spin dried to 8

μL. Then, 20× SSC (2 μL) and dextran sulfate formamide (DS/FA) (10 μL) were added to the spin dried DNA+probe solution to complete the probe mix. The probe mix was added to each slide, covered with a glass cover slip, sealed with photo glue, and incubated in a wet box overnight at 37 °C.

The following day, cover slips were removed and slides were incubated in 2× SSC for five min and 2× SSC (heated to 42 °C) for another 10 min. Next, the slides were passed through successive 5-min incubations of 2× SSC and 1× phosphate-buffered saline (PBS). In the dark, the antibody mix (100 μL per slide) was constructed. The antibody mix was composed of 5× TNB Blocking Buffer (20 μL), filter-sterilized water (80 μL), biotin antibody (1 μL), and digoxigenin antibody (1 μL). The antibody mix was added to each slide, a plastic cover slip was added, and slides were incubated in the dark at 37 °C for 1 h. Next, the plastic cover slips were removed and slides were passed through three successive 5-min incubations in Buffer 1. Slides were removed and Buffer 1 was gently blotted away with a low-lint tissue before adding DAPI mounting medium (20 μL per slide), covering with a glass cover slip, and incubating at 4 °C for 15 min. Then, slides were viewed in the dark on a compound microscope (Axio Imager A1; Zeiss) with a fluorescent light source (X-Cite 120Q; Excelitas Technologies, Waltham, MA). At least five metaphase cells with clear rDNA signals were observed for each taxon. Images of fluorescent DAPI, 5S, and 45S signals were captured separately using a camera attachment (AxioCam MRm; Zeiss) and images were combined using image analysis software (AxioVision; Zeiss).

Results and Discussion

Flow cytometry. Significant differences were found among the taxa investigated for holoploid 2C genome size ($P < 0.0001$). The majority of taxa investigated were tetraploid with holoploid genome sizes ranging between 4.55 ± 0.02 pg in Sugar Tip® to 4.78 ± 0.06 pg in Lil' Kim™ (Table 6.3). No statistical difference in holoploid genome size was found among the tetraploid cultivars (Table 6.3). Our findings for many cultivars agreed with past literature on ploidy levels in *H. syriacus* (Table 6.1). Ploidy and chromosome number was confirmed on the tetraploid group by root tip counts on 'Diana' (4.58 ± 0.02 pg) at $2n = 4x = 80$ (Fig. 6.3), in contrast to previous reports of 'Diana' being a hexaploid (Egolf, 1970; Shim et al., 1993; Van Huylenbroeck et al., 2000). Other putative hexaploids from the USNA were found to be tetraploid in contrast to former reports of hexaploidy (Egolf, 1986, 1988), including a single accession of 'Minerva' (4.61 ± 0.03 pg) and a single accession of 'Aphrodite' (4.66 ± 0.03 pg) (Table 6.3).

Five accessions of *H. syriacus* were found to have genome sizes significantly larger than the tetraploid taxa (Table 6.3). 'Aphrodite', 'Minerva', and all accessions of 'Pink Giant', Azurri Satin®, and Raspberry Smoothie™ proved to be hexaploids (Table 6.3). Holoploid genome sizes ranged from 6.68 ± 0.13 pg in Raspberry Smoothie™ to 7.05 ± 0.18 pg in 'Aphrodite'. Having two cytotypes of 'Aphrodite' and 'Minerva' in the cultivar collection could represent a reversion to the tetraploid state over many years of propagation. However, nursery practices including sexual propagation of cultivars, cultivar substitution, mislabeling, and seedling invasion of stock plants have been shown to degrade cultivar collections in previous studies

(Fantz, 1994). Confirming a previous report by Van Huylenbroeck et al. (2000), 'Pink Giant' was found to be a hexaploid at 6.97 ± 0.07 pg (Table 6.3). In a combined run on a flow cytometer, histograms for the tetraploid Bali™ were clearly distinguishable from the hexaploid 'Pink Giant' (Fig. 6.4A). Flower color and form were similar for hexaploids 'Aphrodite', 'Pink Giant', and 'Minerva' (single, pink flowers). However, Azurri Satin® represents the first single, blue-flowered hexaploid *H. syriacus*, produced by Van Laere et al. (2006). In addition, the pink, double-flowered Raspberry Smoothie™ is the first reported hexaploid, double-flowered *H. syriacus*. Depending on their fertility, Azurri Satin® and Raspberry Smoothie™ may offer new opportunities to breeders seeking novel floral phenotypes to use in interploid crosses.

In addition to tetraploid and hexaploid cytotypes, one accession, Peppermint Smoothie™, was found to be a cytochimera, producing both tetraploid cells (4.61 ± 0.06 pg) and octaploid cells (8.98 ± 0.13 pg) and represents the only mixoploid found (Table 6.3). Histograms from flow cytometry revealed that the tetraploid and octaploid cells could clearly be distinguished and occur at a similar frequency in Peppermint Smoothie™ (Fig. 6.4B). The multiple accessions tested of this cultivar represents the first reported mixoploids for *H. syriacus*.

Ploidy series. After 935 reciprocal pollinations among tetraploid cultivars and 'Pink Giant', 112 capsules were recovered containing a total of 564 seeds. Viable seedlings were recovered from 16 combinations and 'Pink Giant' proved successful as both a seed parent and pollen parent. A subset of these combinations were evaluated with flow cytometry, which revealed numerous seedlings with intermediate

(putative pentaploid) genome sizes (Fig. 6.5). Only one cross, *H. syriacus* 'Woodbridge' x 'Pink Giant', yielded a near-tetraploid average genome size (4.94 ± 0.19 pg) (Fig. 6.5) likely due to high rates of self-pollinations in 'Woodbridge'. However, one seedling (H2012-041-01), resulting from this cross had a hexaploid genome size of 6.78 pg in a single estimate from flow cytometry. Another cross, *H. syriacus* Bali™ x 'Pink Giant' consistently gave pentaploid genome sizes. Four full-sib seedlings were tested using flow cytometry, generating an average genome size for the resulting seedlings of 5.70 ± 0.04 (Fig. 6.5). One vigorous seedling (H2013-078-01), representing this cross was selected for the ploidy series and included in a combined flow cytometry analysis with both of its parents (Fig. 6.4A). This seedling had an intermediate genome size (5.52 pg) and histograms were clearly distinguishable for the seedling and each parent (Fig. 6.4A). Subsequently, three individual analyses using flow cytometry revealed this seedling to have an average holoploid genome size of 5.55 ± 0.02 pg (Table 6.4). This seedling was included as a representative pentaploid in further analysis of stomata anatomy and variation in copy number of rDNA signals.

During the octaploid induction experiment, treatments with SFIs appeared to leave the meristem undamaged and intact during germination and subsequent flushes of new growth. However, several weeks after potting treated seedlings, prior to obtaining mortality data, necrosis began to appear on nearly all seedlings (Fig. 6.1D). Plants produced healthy shoots post treatment only to later spontaneously abscise at the treatment site. Control plants appeared unaffected indicating that treatment with SFIs to young seedlings caused the subsequent tissue death near the treatment site.

After discovering the abscission, cuttings were taken acropetal to the treatment site and rooted under mist. Thirty-four plants were recovered from the autopolyploid induction experiment, representing a fraction of the original seedling population. Prior to potting the cuttings, adventitious roots were collected for analysis via flow cytometry. Adventitious roots provided only enough material for a single run on the flow cytometer per sample without proving too damaging to the young cuttings (Table 6.5).

Of the remaining plants, only two 'Aphrodite' OP seedlings treated with colchicine produced shoots that rooted. Flow cytometry of roots revealed seedling OP2014-27 and OP2014-35 were high level polyploids with holoploid genome sizes of 8.37 pg (8*x*) and 12.22 pg (~10*x*), respectively (Table 6.5). No colchicine-treated seedlings of Bali™ remained for genome size analysis. Only two 10-d and 20-d oryzalin treatments of 'Aphrodite' OP seedlings were recovered for root tip flow cytometry, yielding tetraploid genome sizes (Table 6.5). The remaining 30 accessions were all recovered from 5-d oryzalin treatments, with 18 representing OP seedlings of 'Aphrodite' and 12 representing OP seedlings of Bali™ (Table 6.5). At least one representative from each of the original five replicates was recovered for root tip flow cytometry. For the OP 'Aphrodite' seedlings, 13 seedlings (72%) were found to be ocotoploid with an average genome size of 8.36 ± 0.11 pg (Table 6.5). For the OP Bali™ seedlings, 11 seedlings (92%) were found to be octaploids with an average genome size of 8.36 ± 0.22 pg (Table 6.5). However, few seedlings were recovered from the original experiment and only one sample per seedling was

analyzed using flow cytometry, our results indicate that colchicine and oryzalin provide an efficient way to produce octaploids.

From this autopolyploid population, one OP Bali™ seedling (OP2014-19) from the 5-d oryzalin treatment was selected for further flow cytometry analysis on leaf tissue, yielding an average genome size of 8.88 ± 0.01 pg (Table 6.4). This seedling was selected as a representative ocotoploid for the ploidy series and for further analysis of stomata anatomy and variation in rDNA signals. In a comparison of flow cytometry histograms among ploidy, the octaploid nuclei of OP2014-19 (Fig. 6.4C) can clearly be distinguished from tetraploid, pentaploid, and hexaploid taxa (Fig. 6.4A). In addition, the histogram of OP2014-19 is comparable to the octaploid peak in the mixoploid Peppermint Smoothie™ (Fig. 6.4B).

The ploidy series (4*x*, 5*x*, 6*x*, and 8*x*) was reported for variation in holoploid genome size, stomatal guard cell length, stomatal density, and rDNA signals. Significant differences were found in 2C genome sizes from leaf nuclei among the four taxa investigated ($P < 0.0001$) (Table 6.4). As expected, pairwise comparisons of holoploid genome sizes revealed significant differences among ploidy levels (Table 6.4). Observations of cuticle cells revealed that cell size, including stomata, appeared to increase with increasing ploidy (Fig. 6.6). Measurement of stomatal guard cells found significant differences among the four taxa in the ploidy series ($P < 0.0001$) (Table 6.4). Average guard cell lengths were significantly different for each pairwise comparison and an increase in guard cell size was measured from 27.36 ± 0.04 μm in the tetraploid to 40.48 ± 1.05 μm in the octaploid (Table 6.4).

These results confirm previous reports that stomata size could be useful in determination of ploidy in different species of *Hibiscus* (Contreras et al., 2009; Zhuang and Song, 2005). In contrast to stomata in *H. syriacus*, stomata guard cell lengths reported in *H. schizopetalus, H. mutabilis,* and *H. rosa-sinensis* were much smaller, ranging from 14.26 ± 0.39 μm to 21.10 ± 0.65 μm, respectively (Zhuang and Song, 2005). Essiett and Iwok (2014) reported lengths similar to our measurements in *H. syriacus* for *H. surattensis, H. acetocella, H. rosa-sinensis,* and *H. arnottianus* at 28 μm, 29 μm, 34 μm, and 35 μm, respectively. In induced autotpolyploids of *H. acetocella* 'Panama Red', guard cell size increased from 25 μm in the tetraploid to 36 μm in the octaploid. Our results are also comparable to a previous report on tetraploid and colchicine-induced octaploid *H. syriacus*. Lee and Kim (1976) found that average guard cell lengths of control tetraploid *H. syriacus* ranged from 28.00 μm to 29.10 μm while octaploid guard cell lengths ranged from 31.90 μm to 41.53 μm. A recent study by Li and Ruter (2017) revealed average guard cell lengths in *H. moscheutos* ranging from 24.3 μm for diploids to 31.8 μm for tetraploids (J. Ruter, personal communication). These results combined with the current study indicate that guard cell length may be useful for evaluating both interploid and interspecific hybrids in *Hibiscus*.

Significant differences were found in stomatal densities among the four taxa in the ploidy series ($P = 0.0002$) (Table 6.4). Stomatal densities ranged from 398.22 ± 15.43 stomata·mm^{-2} in the tetraploid to 154.01 ± 8.90 stomata·mm^{-2} in the octaploid. There was a significant, precipitous decline in stomatal density from the tetraploid to pentaploid cytotype representing a two-fold difference in stomata density

(Table 6.4). However, no significant differences in stomatal density were detected among the pentaploid, hexaploid, and octaploid cytotypes. A plot of stomatal guard cells sizes and stomatal densities illustrates the negative relationship between guard cell length and density (Fig. 6.7). The positive relationship between genome size and stomata size, as well as the negative relationship between genome size and stomatal density, proposed by Beaulieu et al. (2008), was observed in the *H. syriacus* ploidy series.

Stomatal index has proven a useful anatomical character for comparing taxa because it is independent of the environment, size, or portion of the leaf surface (Essiett and Iwok, 2014). However, measures differ depending on if total epidermal cell counts are performed, as in Essiett and Iwok (2014), versus the method used in the current study (Li et al., 1996) that solely relies on the product of the stomata length by the stomata density. For the ploidy series in *H. syriacus*, significant differences were found in stomatal indices among the four taxa investigated (P = 0.0008). The strong effect from the change in stomatal density from tetraploid to pentaploid cytotype resulted in the same trend in the pairwise comparisons among ploidy levels (Table 6.4). Polyploids in *Betula* were also found to have smaller stomatal indices, with 4,706 for pentaploids and 5,055 for hexaploids, compared to the diploid controls at 6,103 (Li et al., 1996). Therefore, the reduction in stomatal index in *H. syriacus* high level polyploids may lead to an increase in stress tolerance by reduced transpiration. This effect has been claimed in woody plants, such as *Betula*, but has been illustrated in herbaceous taxa such as the drought tolerant

autopolyploids in *Arabidopsis* (Del Pozo and Ramirez-Parra, 2014) and drought tolerant allopolyploids in wheat (Xiong et al., 2006).

FISH. Across the ploidy series in *H. syriacus*, the number of signals from the 5S rDNA locus varied from two (in the tetraploid) to four (in the octaploid) while signals from the 45S rDNA locus varied from four (in the tetraploid) to eight (in the octaploid) (Fig. 6.8). The use of rDNA signals, combined with flow cytometry, proved useful for confirming ploidy levels in *H. syriacus*, a species with numerous small chromosomes and tolerant of high level polyploids. The discovery of only two 5S rDNA signals in tetraploids (Fig. 6.8A) could provide evidence for *H. syriacus* being a diploid, an allotetraploid with disomic segregation (functional diploid), or an ancestral autopolyploid with subsequent elimination of rDNA sites. However, it is unlikely that *H. syriacus* originated from a recent autopolyploid event, as we would have expected to see at least four 5S rDNA signals.

A recent genome analysis of *H. syriacus* confirms its polyploid status ($2n = 4x = 80$) after multiple occurrences of whole-genome duplication followed by diploidization after speciation (Kim et al., 2017). In addition, allopolyploidy has proved pervasive in other species of *Hibiscus*. Polyploidy has been investigated in *Hibiscus* section *Furcaria* with tetraploids, hexaploids, octaploids, and decaploids all exhibiting allopolyploidy (Menzel and Wilson, 1969; Wilson, 1994, 1999). Tetraploids of section *Furcaria* have been discovered to be allopolyploids including *H. acetosella* (AABB, $2n = 4x = 72$) and *H. radiatus* (AABB, $2n = 4x = 72$) (Satya et al., 2012).

Cotton (*Gossypium*), a close relative to *Hibiscus* in the Malvaceae family, is composed of diploids and allopolyploids. A study of 5S and 45S rDNA revealed that most diploids had two 5S rDNA signals and all allotetraploid species (*G. hirsutum*, *G. barbadense*, *G. tomentosum*, and *G. mustelinum*) had four 5S rDNA signals (Gan et al., 2013), compared to only two 5S rDNA signals in tetraploid *H. syriacus*. In addition to among-ploidy level variation in rDNA signals, it is possible to have variation within the same ploidy for a species. In a comparative analysis of species in the Brassicaceae, species with the same chromosome number were found to have up to a 5-fold difference in rDNA sites (Hasterok et al., 2006). As only one tetraploid of *H. syriacus* was investigated, the background variability in copy number of tetraploid rDNA signals remains to be discovered in althea.

Low copy numbers of 5S rDNA signals are not unique to *H. syriacus*. Elimination of 5S rDNA post polyploidization has been studied in other woody plants such as *Rubus* (Wang et al. 2015B). Both diploid and tetraploid species of *Rubus* carried two 5S sites where triploid and octaploid carried only three (Wang et al. 2015B). This phenomenon is common in FISH studies within the Rosaceae as reported for *Fragaria* (Liu and Davis, 2011), *Prunus* (Maghuly et al., 2010), and *Sanguisorba* (Mishima et al., 2002). However, to our knowledge this is the first report of possible 5S rDNA site elimination in the Hibiscus.

Elimination of 5S rDNA sites in *H. syriacus* could lend evidence to its possible allopolyploid origin. Loss of rDNA has been studied in other allopolyploid taxa such as cotton (Wendel et al., 1995), tobacco (Volkov et al., 1999), *Tragopogon* (Kovarik et al., 2005), *Cardamine* (Franzke and Mummenhoff, 1999) and wheat

(Baum and Feldman, 2010). Drastic genome reorganization and modification often occur in newly formed allopolyploids (Kotseruba et al., 2003). Genes (rDNA) in these allopolypoids likely undergo the process of concerted evolution and interlocus homogenization (Álvarez and Wendel, 2003). These processes can result in significant changes in rDNA sites, including locus loss as demonstrated in the allotetraploid grass *Zingeria* (Kotseruba et al., 2003). Evolution in allopolyploids often results in copy number and transcription changes in rDNA sites through a process of nucleolar dominance first described by Navashin (1934) where rDNA of one parent can be functionally dominant to the other parent in allopolyploids (Pikaard, 2000). Partial or whole copy loss of rDNA sites in allopolyploids has also occurred from suppressed/inactive NORs that prevent normal replication leading to stepwise elimination of rDNA as observed in cereals (Dvorak, 1990; Gustafson et al., 1988) and tobacco (Volkov et al., 1999). In addition, elimination of rDNA can occur rapidly in newly formed allopolyploids. Baum and Feldman (2010) found that in wheat, elimination of 5S rDNA occurred within the first three generations after the formation of allopolyploids.

In the current study, putative pentaploids were reported for variation in 5S and 45S copy number. After interploid hybridization, putative pentaploid seedlings were selected for FISH analysis by flow cytometry of single leaf samples, which produced a range of intermediate genome sizes from 5.60 pg to 6.31 pg. (Table 6.6). To our knowledge, these selections represent the first FISH analysis on pentaploids from an interploid cross. Studies on species with natural ploidy series, such as 4*x*, 5*x*, and 6*x* buffelgrass (*Cenchrus ciliaris*) (Kharrat-Souissi et al., 2012), have shown

proportional increases of rDNA signals associated with each ploidy level. In contrast, a non-random distribution of interploid hybrid chromosomes and rDNA sites have been demonstrated in interspecific triploid hybrids of *Epidendrum* where all triploids exhibited the same number of 5S and 45S signals as the diploid parent (Moraes et al., 2013). However, segregation patterns of rDNA signals in *H. syriacus* appeared to be random among the putative pentaploids and signals for each rDNA site ranged between the values of the two parents (Table 6.6).

From the 12 taxa investigated, all possible parental combinations of rDNA signals were found (Fig. 6.9). No obvious pattern was observed between parental combinations or genome size estimates and number of rDNA signals (Table 6.6). From the 12 taxa investigated, two groups of full-sib seedlings were evaluated. From the cross 'Aphrodite' x 'Pink Giant', three seedlings exhibited two combinations of rDNA signals: [H2013-017-11 = two 5S + four 45S] and [H2013-017-21, H2013-077-05 (reciprocal) = three 5S + five 45S]. From the cross 'Helene' x 'Pink Giant', two seedlings exhibited two combinations of rDNA signals: [H2013-124-19 = two 5S + five 45S] and [H2013-124-03 = three 5S + four 45S].

The current study represents an investigation on genome size and ploidy variation in cultivated althea. The majority of cultivars screened were tetraploid, with several taxa confirmed as hexaploid. Raspberry Smoothie™ is reported as a hexaploid for the first time and represents the first identification of a double-flowered hexaploid. Future work will focus on interploid hybridization with Raspberry Smoothie™ to develop odd ploidy level plants with petaloid stamens. The combination of double flowers with odd ploidy may lead to extremely low fertility

selections after the first generation of crossing. One cytochimera, Peppermint Smoothie™, is reported for the first time and future work will focus on determining the ploidy of its pollen grains. If the L-II histogenic layer of Peppermint Smoothie™ represents the octaploid cytotype, then this taxa could be the first double-flowered, functional octaploid for use in future interploid breeding. In contrast to these new cultivars, accessions of an older, previously confirmed hexaploid, 'Aphrodite' and 'Minerva', were found to be both hexaploid and tetraploid while 'Diana' was found to be tetraploid. This result illustrates the necessity for ploidy confirmation of clonal nursery material.

Vigorous plants were recovered from each of the four ploidy levels in our series of *H. syriacus*. The role of polyploidy on plant morphology and physiology has been studied mainly in natural allopolyploids that have extinct diploid progenitors. The consequences of long spans of evolution following ancient hybridization and whole genome duplication can obscure the role of polyploidy in variable morphology and physiology (Soltis et al., 2016). The development of vigorous taxa representing an inter-related, wide ploidy series in *H. syriacus* could prove useful in studying the effects of polyploidy on temperate shrubs. Clonal replicates of each taxon in the ploidy series will be reproduced and mature plants will be used for important measurements of morphological, anatomical, and physiological characteristics such as photosynthetic rate, drought tolerance, cold tolerance, pollen diameter, and fertility. Similar research has recently been performed on ploidy series in *Brassica* (Baker et al., 2017) and *Arabidopsis* (Del Pozo and Ramirez-Parra, 2014). However, our ploidy series contains more cytotypes than the few studies on

polyploidy and abiotic stress response in woody plants, such as *Lonicera* (Li et al., 2009) and *Prunus* (Pustovoitova et al., 1996). Woody, deciduous shrubs with a wide range of ploidy provide a unique opportunity to study winter cold tolerance in relation to ploidy, a topic rarely studied in current literature.

Since colonial times, althea has spread into temperate gardens from coast to coast. The popularity of this shrub with its bright summer blooms will likely remain undiminished in the years to come. For both its potential scientific contributions and for the next generation of improved cultivars for the landscape, our work demonstrates that *H. syriacus* offers a bright future for plant scientists and woody plant breeders.

Literature Cited

Álvarez, I. and J.F. Wendel. 2003. Ribosomal ITS sequences and plant phylogenetic inferences. Mol. Phylogenet. Evol. 29:417-434.

Anamthawat-Jónsson, K. 2003. Preparation of chromosomes from plant leaf meristems for karyotype analysis and in situ hybridization. Methods Cell Sci. 25:91-95.

Bajer, A.S. and J. Molebajar. 1986. Drugs with colchicine-like effects that specifically disassemble plant but not animal microtubules. Ann. New York Acad. Sci. 466:767–784.

Bates, D.M. 1965. Notes on the cultivated Malvaceae. I. *Hibiscus*. Baileya 13: 57-130.

Baum, B.R. and M. Feldman. 2010. Elimination of 5S DNA unit classes in newly formed allopolyploids of the genera *Aegilops* and *Triticum*. Genome 53: 430-438.

Beaulieu, J.M., I.J. Leitch, S. Patel, A. Pendharkar, and C.A. Knight. 2008. Genome size is a strong predictor of cell size and stomatal density in angiosperms. New Phytol. 179:975-986.

Blakeslee, A.F. and A.G. Avery. 1937. Methods of inducing doubling of chromosomes in plants by treatment with colchicine. J. Hered. 28:393-411.

Buechler, W.K., 2000. Estimating polyploidy levels using cell size in *Salix* leaves. Amer. J. Bot. 87:238.

Chang, Y.-C., C.-T. Shii, and M.-C. Chung. 2009. Variations in ribosomal RNA gene loci in spider lily (*Lycoris* spp.). J. Amer. Soc. Hort. Sci. 134:567-573.

Chen, G., W.-B. Sun, and H. Sun. 2009. Morphological characteristics of leaf epidermis and size variation of leaf, flower and fruit in different ploidy levels in *Buddleja macrostachya* (Buddlejaceae). J. System. Evol. 47:231-236.

Chen, H., M.-C. Chung, Y.-C. Tsai, F.-J. Wei, J.-S. Hsieh, and Y.-I.C. Hsing. 2015. Distribution of new satellites and simple sequence repeats in annual and perennial *Glycine* species. Bot. Studies 56:22. DOI: 10.1186/s40529-015-0103-9.

Chung, M.-C., Y.-I. Lee, Y.-Y. Cheng, Y.-J. Chou, and C.-F. Lu. 2008. Chromosomal polymorphism of ribosomal genes in the genus *Oryza*. Theor. Appl. Genet. 116:745-753.

Del Pozo, J.C. and E. Ramirez-Parra. 2014. Deciphering the molecular bases for drought tolerance in *Arabidopsis* autotetraploids. Plant Cell Environ. 37:2722-2737.

Dhooge, E., K.V. Laere, T. Eeckhaut, L. Leus, and J.V. Huylenbroeck. 2010. Mitotic chromosome doubling of plant tissues in vitro. Plant Cell Tissue Organ Cult. 104:359-373.

Dvorak, J. 1990. Evolution of multigene families: the ribosomal RNA loci of wheat and related species. Pp. 83-97 in Plant population genetics, breeding, and genetic resources. Sinauer Associates, Sunderland, Massachusetts.

Eeckhaut, T.G.R., J.M. Van Huylenbroeck, J. De Riek, and E. Van Bockstaele. 2004. Interspecific hybridization between *Hibiscus syriacus* L. and *Hibiscus paramutabilis* Bailey. Acta Hort. (ISHS) 630:85-90.

Egolf, D.R. 1970. *Hibiscus syriacus* ‘Diana’, a new cultivar. Baileya 17:75-78.

Egolf, D.R. 1981. ‘Helene’ rose of sharon (althea). HortScience 16:226-227.

Egolf, D.R. 1986. ‘Minerva’ rose of sharon (althea). HortScience 21:1463-1464.

Egolf, D.R. 1988. ‘Aphrodite’ rose of sharon (althea). HortScience 23:223.

Essiett, U.A. and E.S. Iwok. 2014. Floral and leaf anatomy of *Hibiscus* species. Amer. J. Medical Biol. Res. 2014 2:101-117.

Fantz, P.R. 1994. Taxonomic problems in cultivated liriopogons. HortTechnology 3:146-150.

Franzke, A. and K. Mummenhoff. 1999. Recent hybrid speciation in *Cardamine* (Brassicaceae) – conversion of nuclear ribosomal ITS sequences in statu nascendi. Theor. Appl. Genet. 98:831-834.

Fryxell, 1988. Malvaceae of Mexico. System. Bot. Monogr. 25:1-522.

Gan, Y., F. Liu, D. Chen, Q. Wu, Q. Qin, C. Wang, S. Li, X. Zhang, Y. Wang, and K. Wang. 2013. Chromosomal locations of 5S and 45S rDNA in *Gossypium* genus and its phylogenetic implications revealed by FISH. PLoS ONE 8:e0068207.

Gerlach, W.L. and J.R. Bedbrook. 1979. Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucl. Acids Res. 7:1869–1885.

Gerlach, W.L. and T.A. Dyer. 1980. Sequence organization of the repeated units in the nucleus of wheat which contain 5S rRNA genes. Nucl. Acids Res. 8:4851–4865.

Gomes, S.S.L., C.W. Saldanha, C.S. Neves, M. Trevizani, N.R.B. Raposa, M.M. Notini, M.O. Santos, J.M.S. Campos, W.C. Otoni, and L.F. Viccini. 2014.

Karyotype, genome size, and in vitro chromosome doubling of *Pfaffia glomerata* (Spreng.) Pedersen. Plant Cell. Tissue Organ Cult. 118:45-56.

Greilhuber, J., J. Doležel, M.A. Lysák, and M.D. Bennett. 2005. The origin, evolution and proposed stabilization of the terms 'genome size' and 'C-value' to describe nuclear DNA contents. Ann. Bot. 95:255–260.

Gustafson, J.P., A.R. Dera, and S. Petrovic. 1988. Expression of modified rye ribosomal RNA genes in wheat. Proc. Natl. Acad. Sci. USA 85:3943-3945.

Hasterok, R., E. Wolny, M. Hosiawa, M. Kowalczyk, S. Kulak-Ksiazczyk, T. Ksiazczyk, W.K. Heneen, and J. Maluszynska. 2006. Comparative analysis of rDNA distribution in chromosomes of various species of Brassicaceae. Ann. Bot. 97: 205-216.

Hugdahl, J.D. and L.C. Morejohn. 1993. Rapid and reversible high affinity binding of the dinitroaniline herbicide oryzalin to tubulin from *Zea mays* L. Plant Physiol. 102:725–740

Joly, S. and A. Bruneau. 2007. Delimiting species boundaries in *Rosa* Sect. Cinnamomeae (Rosaceae) in eastern North America. System. Bot. 32:819-836.

McGoey, B.V., K. Chau, and T.A. Dickenson. 2014. Stomata size in relation to ploidy level in North American hawthorns (*Crataegus*, Rosaceae). Madroño 61:177-193.

Kharrat-Souissi, A., S. Siljak-Yakovlev, F. Pustahija, and M. Chaieb. 2012. Physical mapping of 5S and 18S-5.8S-26S RNA gene families in polyploid series of

Cenchrus ciliaris Linnaeus, 1771 (Poaceae). Comparative Cytogenenet. 6:273-286.

Kim, Y.-M., S. Kim, N. Koo, A.-Y. Shin, S.-I. Yeom, E. Seo, S.-J. Park, W.-H. Kang, M.-S. Kim, J. Park, I. Jang, P.-G. Kim, I. Byeon, M.-S. Kim, J. Choi, G. Ko, J. Hwang, T.-J. Yang, S.-B. Choi, J.M. Lee, K.-B. Lim, J. Lee, I.-Y. Choi, B.-S. Park, S.-Y. Kwon, D. Choi, and R.W. Kim. 2017. Genome analysis of *Hibiscus syriacus* insights of polyploidization and indeterminate flowering in woody plants. DNA Res. 24:71-80.

Kotseruba, V. D. Gernand, A. Meister, and A. Houben. 2003. Uniparental loss of ribosomal DNA in the allotetraploid grass *Zingeria trichopoda* (2*n* = 8). Genome 46:156-163.

Kovarik, A., J.C. Pires, A.R. Leitch, K.Y. Leitch, K.Y. Lim, A.M. Sherwood, R. Matyasek, J. Rocca, D.E. Soltis, and P.S. Soltis. 2005. Rapid concerted evolution of nuclear ribosomal DNA in two *Tragopogon* allopolyploids of recent and recurrent origin. Genetics 169:931-944.

Lattier, J.D., H. Chen, and R.N. Contreras. 2017. Improved method of enzyme digestion for root tip cytology. HortScience. (In Press).

Lattier, J.D., T.G. Ranney and N.P. Lynch. 2013. History and cytological reassessment of *Rhododendron canadense*. J. Am. Rhododendron Soc. 67: 92-98.

Lee, S.K. and C.S. Kim. 1976. Studies on artificial polyploidy forest trees XIII: Some morphological and physiological characteristics of colchitetraploid *Hibiscus syriacus* L. J. Korean For. Soc. 32:73-86.

Li, W.L., G.P. Berlyn, and P.M.S. Ashton. 1996. Polyploids and their structural and physiological characteristics relative to water deficit in *Betula papyrifera* (Betulaceae). Amer. J. Bot. 83: 15-20.

Li, Z. and J.M. Ruter. 2017. Development and evaluation of diploid and polyploid *Hibiscus moscheutos*. HortScience 52:676-681.

Li, W.-D., D.K. Biswas, H. Xu, C.-Q. Xu, X.-Z. Wang, J.-K. Liu, and G.-M. Jiang. 2009. Photosynthetic responses to chromosome doubling in relation to leaf anatomy in *Lonicera japonica* subjected to water stress. Functional Plant Biol. 36:783-792.

Liu B. and T.M. Davis. 2011. Conservation and loss of ribosomal RNA gene sites in diploid and polyploid *Fragaria* (Rosaceae). BMC Plant Biol. 11:157.

Maghuly F., B. Schmoellorl, E.M. Temsch, and M. Laimer. 2010. Genome size, karyotyping and FISH physical mapping of 45S and 5S genes in two cherry rootstocks: *Prunus subhirtella* and *Prunus incisa* × *serrula*. J. Biotechnol. 149:88–94.

Menzel, M.Y. and E.D. Wilson. 1969. Genetic relationships in *Hibiscus* sect. *Furcaria*. Brittonia 21:91-125.

Mishima, M., N. Ohmido, K. Fukui, and T. Yahara. 2002. Trends in site-number change of rDNA loci during polyploid evolution in *Sanguisorba* (Rosaceae). Chromosoma. 110:550–558.

Moraes, A.P., M. Chinaglia, C. Palma-Silva, and F. Pinheiro. 2013. Interploidy hybridization in sympatric zones: the formation of *Epidendrum fulgens* × *E.*

puniceoluteum hybrids (Epidendroideae, Orchidaceae). Ecol. Evol. 3: 3824-3837.

Morejohn, L.C., T.E. Bureau, J. Molebajer, A.S. Bajer, and D.E. Fosket. 1987. Oryzalin, a dinitroaniline herbicide, binds to plant tubulin and inhibits microtubule polymerization in vitro. Planta 172:252–264.

Morejohn, L.C., T.E. Bureau, L.P. Tocchi, and D.E. Fosket. 1984. Tubulins from different higher-plant species are immunologically non-identical and bind colchicine differently. Proc. Natl. Acad. Sci. USA 81:1440-1444.

Navashin, M. 1934. Chromosomal alterations caused by hybridization and their bearing upon certain general genetic problems. Cytologia 5:169-203.

Ochatt, S.J. 2008. Flow cytometry in plant breeding. Cytometry 73: 581-598.

Padoan, D., A. Mossad, B. Chiancone, M.A. Germana, and P.S.S.V. Khan. 2013. Ploidy levels in Citrus clementine affects leaf morphology, stomatal density and water content. Theor. Expt. Plant Physiol. 25:283-290.

Pikaard, G.S. 2000. The epigenetics of nucleolar dominance. Trends Genet. 16:495-500.

Prado, E. P. Faivre-Rampant, C. Schneider, and M.A. Darmency. 1996. Detection of a variable number of ribosomal DNA loci by fluorescent in situ hybridization in *Populus* species. Genome 39:1020-1026.

Purdue University Cytometry Laboratories (PUCL). 2014. http://www.cyto.purdue.edu/Purdue_software

Pustovoitova, T.N., G.V. Eremin, E.G. Rassvetaeva, and V.N. Zholkevich. 1996. Drought resistance, recovery capacity, and phytohormone content in polyploid plum leaves. Russian J. Plant Physiol. 43:232-235.

Ribeiro, T. A. Barão, W. Viegas, and L. Morais-Cecílio. 2008. Molecular cytogenetics of forest trees. Cytogenet. Genome Res. 120:220-227.

Satya, P., M. Karan, D. Sarkar, and M.K. Sinha. 2012. Genome synteny and evolution of AABB allotetraploids in *Hibiscus* section *Furcaria* revealed by interspecific hybridization, ISSR and SSR markers. Plant System. Evol. 298:1257-1270.

Saltonstall, K., K. Glennon, A. Burnett, R.B. Hunter, and K.L. Hunter. 2007. Comparison of morphological variation indicative of ploidy level in *Phragmites australis* (Poaceae) from eastern North America. Rhodora 109:415-429.

Shim, K.K., K.H. Kim, and Y.M. Ha. 1993. Characteristics of triploid cultivars ‘Diana’ and ‘Helene’ in *Hibiscus syriacus* L. J. Korean Soc. Hort. Sci. 34:54-67.

Soltis, D.E., C.J. Visger, D.B. Marchant, and P.S. Soltis. 2016. Polyploidy: Pitfalls and paths to a paradigm. Amer. J. Bot. 103:1-21.

Srivastava, A.K. and D. Schlessinger. 1991. Structure and organization of ribosomal DNA. Biochimie 73: 631-638.

Van Huylenbroeck, J.M., J. De Riek, and M. De Loose. 2000. Genetic relationships among *Hibiscus syriacus*, *Hibiscus sinosyriacus* and *Hibiscus paramutabilis*

revealed by AFLP, morphology and ploidy analysis. Genet. Res. Crop Evol. 47:335-343.

Van Laere, K., J. Van Huylenbroeck, and E. Van Bockstaele. 2006. Breeding strategies for genetic variability within *Hibiscus syriacus*. Acta Hort. (ISHS) 714:75-81.

Van Laere, K., J.M. Van Huylenbroeck, and E. Van Bockstaele. 2007. Interspecific hybridization between *Hibiscus syriacus*, *Hibiscus sinosyriacus*, and *Hibiscus paramutabilis*. Euphytica 155:271-283.

Van Laere, K., A. Dewitte, J. Van Huylenbroeck, and E. Van Bockstaele. 2009. Evidence for the occurrence of unreduced gametes in interspecific hybrids of *Hibiscus*. J. Hort. Sci. Biotechnol. 84:240-247.

Volkov, R.A., N.V. Borisjuk, I.I. Panchuk, D. Schweizer, and V. Hemleben. 1999. Elimination and rearrangement of parental rDNA in the allotetraploid *Nicotiana tobacum*. Mol. Biol. Evol. 16:311-320.

Wang, Q., J. Wang, Y. Zhang, Y. Zhang, S. Xu, and Y. Lu. 2015A. The application of fluorescent in situ hybridization in different ploidy levels cross-breeding of lily. PLoS ONE 10: e0126899.

Wang, Y., X. Wang, Q. Chen, L. Zhang, H. Tang, Y. Luo and Z. Liu. 2015B. Phylogenetic insights into subgenera *Idaeobatus* and *Malachobatus* (*Rubus*, Rosaceae) inferring from ISH analysis. Mol. Cytogenet. 8:11. Doi: 10.1186/s13039-015-0114-y

Wendel, J.F., A. Schnabel, and T. Seelanan. 1995. Bidirectional interlocus concerted evolution following allopolyploid speciation in cotton (*Gossypium*). Proc. Natl. Acad. Sci. 92:280-284.

Wilson, E.D. 1994. The genome biogeography of *Hibiscus* L. section *Furcaria* DC. Genet. Res. Crop Evol. 41:13-25.

Wilson, E.D. 1999. Revision of *Hibiscus* section *Furcaria* (Malvaceae) in Africa and Asia. Bul. Natl. History Museum London Bot. 29:47-79.

Xiong, Y.-C., F.-M. Li, and T. Zhang. 2006. Performance of wheat crops with different chromosome ploidy: root-sourced signals, drought tolerance, and yield performance. Planta 224:710-718.

Zhuang, D.-H. and J.-J. Song. 2005. The characters of pollen grains and stomatal apparatus in *Hibiscus* L. in relation to ploidy. J. Trop. Subtrop. Bot. 13: 49-52.

Tables

Table 6.1. Previous reports of ploidy in cultivars of *Hibiscus syriacus*.

Publication	Cultivar, trademark, or accession	Reported ploidy[z]	Adjusted ploidy[y]
Egolf, 1970			
	'Diana'	3*x*	6*x**
Lee and Kim, 1976			
	'Sp_1'	4*x*	8*x*
	'Sp_2'	4*x*	8*x*
	'Sd_1'	4*x*	8*x*
Egolf, 1981			
	'Helene'	3*x*	6*x**
Egolf, 1986			
	'Minerva'	3*x*	6*x**
Egolf, 1988			
	'Aphrodite'	3*x*	6*x**
Shim et al., 1993			
	'Purple CV_2'	4*x*	8*x**
	'Red Heart CV'	4*x*	8*x**
	'Diana'	3*x*	6*x**
	'Helene'	3*x*	6*x**
	'Blue Bird' (Oiseau Bleu)	2*x*	4*x**
	'Hansarang'	2*x*	4*x**
	'Youngkwang'	2*x*	4*x**
Van Huylenbroeck et al., 2000			
	'Purple CV_2'	4*x*	8*x**
	'Red heart CV'	4*x*	8*x**
	'Diana'	3*x*	6*x**
	'Helene'	3*x*	6*x**
	'Melrose' (Pastello)	3*x*	6*x**
	'Pink Giant'	3*x*	6*x**
	'Shimsan'	3*x*	6*x**
	'Admiral Dewey'	2*x*	4*x**
	'Amplissimus'	2*x*	4*x**
	'Ardens'	2*x*	4*x**
	'Boule de Feu'	2*x*	4*x**
	'Carneus Plenus'	2*x*	4*x**
	'Coelestis'	2*x*	4*x**
	'Coeruleus Plenus'	2*x*	4*x**
	'Collie Mullens'	2*x*	4*x**
	'Comte de Hainaut'	2*x*	4*x**

Table 6.1 (continued). Previous reports of ploidy in cultivars of *Hibiscus syriacus*.

Publication	Cultivar, trademark, or accession	Reported ploidy[z]	Adjusted ploidy[y]
	'Dorothy Crane'	2*x*	4*x**
	'Duc de Brabant'	2*x*	4*x**
	'Hamabo'	2*x*	4*x**
	'Jeanne d'Arc'	2*x*	4*x**
	'Lady Stanley' (Elegantissimus)	2*x*	4*x**
	'Leopoldii'	2*x*	4*x**
	'Lucy'	2*x*	4*x**
	'Marina' Blue Satin®	2*x*	4*x**
	'Mathilde' Blush Satin®	2*x*	4*x**
	'Mauve Queen'	2*x*	4*x**
	'Meehanii'	2*x*	4*x**
	'Melwhite' (Bianco)	2*x*	4*x**
	'Monstrosus'	2*x*	4*x**
	'Blue Bird' (Oiseau Bleu)	2*x*	4*x**
	'Puniceus Plenus'	2*x*	4*x**
	'Purpureus Variegatus'	2*x*	4*x**
	'Red Heart'	2*x*	4*x**
	'Roseus Plenus'	2*x*	4*x**
	'Ruber Plena'	2*x*	4*x**
	'Rubis'	2*x*	4*x**
	'Floru' Russian Violet®	2*x*	4*x**
	'Souvenir de Charles Breton'	2*x*	4*x**
	'Speciosus'	2*x*	4*x**
	'Totus Albus'	2*x*	4*x**
	'Violet Clair Double'	2*x*	4*x**
	'William R. Smith'	2*x*	4*x**
	'Woodbridge'	2*x*	4*x**
Eeckhaut et al., 2004			
	'Purple CV_2'	4*x*	8*x**
	'Red Heart CV'	4*x*	8*x**
	'Melwhite' (Bianco)	2*x*	4*x**
	'Blue Bird' (Oiseau Bleu)	2*x*	4*x**
Van Laere et al., 2006			
	'Purple CV'	8*x*	8*x*
	'Red Heart CV'	8*x*	8*x*
	'Marina' Blue Satin®	4*x*	4*x*
	'Melwhite' (Bianco)	4*x*	4*x*
	'Blue Bird' (Oiseau Bleu)	4*x*	4*x*
	'Woodbridge'	4*x*	4*x*

Table 6.1 (continued). Previous reports of ploidy in cultivars of *Hibiscus syriacus*.

Publication	Cultivar, trademark, or accession	Reported ploidy[z]	Adjusted ploidy[y]
Van Laere et al., 2007			
	'Purple CV_2'	8*x*	8*x*
	'Red Heart CV'	8*x*	8*x*
	'Freedom'	4*x*	4*x*
	'Melwhite' (Bianco)	4*x*	4*x*
	'Blue Bird' (Oiseau Bleu)	4*x*	4*x*
Van Laere et al., 2009			
	'Red Heart CV'	8*x*	8*x*
	'Blue Bird' (Oiseau Bleu)	4*x*	4*x*

[z]Published ploidy level

[y]Actual ploidy level or adjusted ploidy (*) based on Skovsted (1941) report that *H. syriacus* wild cytotype is tetraploid.

Table 6.2. Source material for *Hibiscus syriacus*.

Cultivar[z]	Trade name[y]	Accession[x]	Source[w]
'American Irene Scott'	Sugar Tip®	12-0019	Bailey Nurseries
'Antong Two'	Lil' Kim™	12-0021	Bailey Nurseries
'Aphrodite'		13-0054	Monrovia
		11-0215	Bailey Nurseries
'Ardens'		13-0050	Blue Heron
'Oiseau Bleu'	'Blue Bird'	11-0219	Monrovia
		13-0057	Monrovia
'Blushing Bride'		13-0048	Blue Heron
		13-0059	Monrovia
'Bricutts'	China Chiffon™	13-0060	Monrovia
'Buddha Belly'		14-0128	Yamaguchi Nursery
'Collie Mullins'		13-0061	Monrovia
'Diana'		13-0062	Monrovia
		11-0211	Bailey Nurseries
'DS01BS'	Blueberry Smoothie™	14-0092	Greenleaf Nursery
'DS02SS'	Strawberry Smoothie™	14-0091	Greenleaf Nursery
'DS03RS'	Raspberry Smoothie™	14-0094	Greenleaf Nursery
'DS04PS'	Peppermint Smoothie™	14-0093	Greenleaf Nursery
'DVPazurri'	Azurri Satin®	13-0055	Monrovia
		14-0188	Spring Meadow
		16-0015	Forestfarm
'Pink Giant'		11-0217	Bailey Nurseries
'Floru'	Violet Satin®	13-0118	JC Raulston Arboretum
		13-0119	JC Raulston Arboretum
'Helene'		13-0063	Monrovia
		13-0116	JC Raulston Arboretum
		13-0117	JC Raulston Arboretum
'JWNfour'	Pink Chiffon®	13-0067	Monrovia
'Lucy'		11-0216	Bailey Nurseries
'Marina'	Blue Satin®	13-0094	JC Raulston Arboretum
		11-0210	Bailey's Nursery
'Mathilde'	Blush Satin®	13-0058	Monrovia
'Mineru'	First Editions® Tahiti™	12-0024	Bailey Nurseries
		13-0098	Bailey Nurseries
'Minerva'		13-0051	Blue Heron
		13-0066	Monrovia
		11-0213	Bailey Nurseries
'Minfren'	First Editions® Bali™	12-0023	Bailey Nurseries
'Minrosa'	Rose Satin®	13-0068	Monrovia
		13-0068	Monrovia

Table 6.2 (continued). Source material for *Hibiscus syriacus*.

Cultivar[z]	Trade name[y]	Accession[x]	Source[w]
'Minspot'	First Editions® Fiji™	12-0022	Bailey Nurseries
'Minsygrbl1'	First Editions® Hawaii™	12-0020	Bailey Nurseries
		13-0096	Bailey Nurseries
'Notwoodone'	Lavender Chiffon™	13-0046	Blue Heron
		13-0064	Monrovia
'Notwoodthree'	Blue Chiffon™	13-0056	Monrovia
		11-0218	Blue Heron
'Notwoodtwo'	White Chiffon®	13-0044	Blue Heron
'Red Heart'		13-0049	Blue Heron
'Woodbridge'		11-0214	Bailey Nurseries
		13-0047	Blue Heron

[z]Cultivar name.

[y]Trademark name.

[x]Accession number in research collection at the Ornamental Plant Breeding Lab, Oregon State University, Corvallis, OR.

[w]Container plant collected from the following sources: Bailey Nurseries, Yamhill, OR; Blue Heron Farm, Corvallis, OR; Forestfarm Nursery, Williams, OR; Greenleaf Nursery, Park Hill, OK; JC Raulston Arboretum, Raleigh, NC; Monrovia, Dayton, OR; Spring Meadow Nursery, Grand Haven, MI; Yamaguchi Plantsman Nursery, Gifu, Japan.

Table 6.3. Ploidy and relative 2C genome size in althea (*Hibiscus syriacus* L.).

Cultivar / trade name[z]	Accession[y]	Ploidy[x]	2C genome size (pg)[w]
'Aphrodite'	11-0215[v]	6*x*	7.05 ± 0.18 A
'Pink Giant'	11-0217	6*x*	6.97 ± 0.07 AB
'Minerva'	13-0051	6*x*	6.86 ± 0.03 AB
Azurri Satin®	14-0188	6*x*	6.81 ± 0.04 AB
Raspberry Smoothie™	14-0094	6*x*	6.68 ± 0.13 B
Lil' Kim™	12-0021	4*x*	4.78 ± 0.06 C
Strawberry Smoothie™	14-0091	4*x*	4.73 ± 0.07 C
'Woodbridge'	11-0214	4*x*	4.68 ± 0.04 C
'Blushing Bride'	13-0059	4*x*	4.68 ± 0.04 C
'Ardens'	13-0050	4*x*	4.67 ± 0.05 C
White Chiffon®	13-0044	4*x*	4.66 ± 0.04 C
'Aphrodite'	11-0215[v]	4*x*	4.66 ± 0.03 C
Tahiti™	12-0024	4*x*	4.66 ± 0.06 C
Blush Satin®	13-0058	4*x*	4.66 ± 0.03 C
'Buddha Belly'	14-0128	4*x*	4.66 ± 0.05 C
Lavender Chiffon™	13-0046	4*x*	4.64 ± 0.03 C
Blue Chiffon™	13-0056	4*x*	4.63 ± 0.02 C
'Collie Mullins'	13-0061	4*x*	4.63 ± 0.03 C
'Bluebird'	11-0219	4*x*	4.63 ± 0.04 C
Fiji™	12-0022	4*x*	4.63 ± 0.03 C
Blue Satin®	11-0210	4*x*	4.62 ± 0.03 C
'Lucy'	11-0216	4*x*	4.62 ± 0.04 C
Hawaii™	12-0020	4*x*	4.62 ± 0.04 C
Bali™	12-0023	4*x*	4.61 ± 0.00 C
'Minerva'	11-0213	4*x*	4.61 ± 0.03 C
Peppermint Smoothie™	14-0093	4*x* + 8*x*	4.61 ± 0.06 C | 8.98 ± 0.13
Blueberry Smoothie™	14-0092	4*x*	4.60 ± 0.04 C
'Red Heart'	11-0212	4*x*	4.60 ± 0.03 C
Pink Chiffon®	13-0067	4*x*	4.60 ± 0.04 C
China Chiffon™	13-0060	4*x*	4.59 ± 0.04 C
'Diana'	11-0211	4*x*[u]	4.58 ± 0.02 C
Sugar Tip®	12-0019	4*x*	4.55 ± 0.02 C

[z]Cultivar or trademark name.
[y]Accession number in the Ornamental Plant Breeding Lab at Oregon State University.
[x]Ploidy level.
[w]2C holoploid genome size; minimum significant difference of 0.3103 based on Tukey's honestly significant difference test.
[v]Multiple replicates from the same source under the same accession number.
[u]Chromosomes counted using root tip cytology

Table 6.4. Relative genome size and stomata anatomy in a ploidy series of *Hibiscus syriacus*.

Ploidy[z]	2C genome size (pg)[y]	Guard cell length (µm) [x]	Stomatal density (stomata·mm^{-2})[w]	Stomatal index[v]
Tetraploid	4.61 ± 0.00 A[t]	27.36 ± 0.04 A[t]	398.22 ± 15.43 A[t]	10,894.45 ± 411.95 A[t]
Pentaploid	5.55 ± 0.02 B	30.35 ± 1.28 B	194.06 ± 38.69 B	5,810.05 ± 995.15 B
Hexaploid	6.97 ± 0.07 C	35.59 ± 0.63 C	180.61 ± 13.14 B	6,411.81 ± 355.68 B
Octaploid	8.88 ± 0.01 D	40.48 ± 1.05 D	154.01 ± 8.90 B	6,215.92 ± 196.89 B

[z]Ploidy series including tetraploid (*H. syriacus* Bali™ 12-0023), pentaploid (F_1 hybrid 'Pink Giant' × Bali™ H2013-078-01), hexaploid (*H. syriacus* 'Pink Giant' 11-0217), and octaploid (open-pollinated, oryzalin-treated seedling from *H. syriacus* Bali™ OP2014-19).

[y]Holoploid, 2C relative genome sizes [mean ± SEM (pg)].

[x]Length of guard cells measured at magnification ×200 [mean ± SEM (µm)].

[w]Number of stomata × 6.62 at magnification ×200 [mean ± SEM (stomata·mm^{-2})].

[v]Stomata index = stomatal density × guard cell length [mean ± SEM].

[t]Means represent averages of three replicates separated within columns based on Fisher's least significant difference [α<0.05]. Minimum significant differences for each column (left to right) is 0.1156 pg, 2.8892 µm, 72.68 stomata·mm^{-2}, and 1877.2 (stomatal index).

Table 6.5. Flow cytometry on roots of colchicine and oryzalin treated open-pollinated seedlings of *Hibiscus syriacus*.

Open-pollenated seedling[z]	Chemical treatment[y]	Duration (days)	Replicate (no.)[x]	Accession (no.)	Holoploid 2C genome size (pg)[w]	Ploidy estimate[v]
'Aphrodite'	Colchicine	10 days	Rep 4	OP2014-27	8.37	7.2*x*
			Rep 5	OP2014-35	12.22	10.5*x*
	Oryzalin	10 days	Rep 1	OP2014-11	4.61	4.0*x*
		20 days	Rep 4	OP2014-30	4.61	4.0*x*
		5 days	Rep 1	OP2014-10	8.68	7.5*x*
				OP2014-16	7.44	6.4*x*
			Rep 2	OP2014-29	8.96	7.7*x*
				OP2014-15	4.62	4.0*x*
			Rep 3	OP2014-13	8.63	7.4*x*
				OP2014-32	8.38	7.2*x*
				OP2014-33	8.17	7.0*x*
				OP2014-23	7.77	6.7*x*
				OP2014-07	4.48	3.9*x*
			Rep 4	OP2014-04	8.53	7.4*x*
				OP2014-09	8.51	7.3*x*
				OP2014-02	4.56	3.9*x*
				OP2014-06	4.49	3.9*x*
			Rep 5	OP2014-12	8.54	7.4*x*
				OP2014-05	8.44	7.3*x*
				OP2014-20	8.42	7.3*x*
				OP2014-17	8.25	7.1*x*
				OP2014-08	4.49	3.9*x*
Bali™	Oryzalin	5 days	Rep 1	OP2014-18	8.12	7.0*x*
				OP2014-25	7.99	6.9*x*
			Rep 2	OP2014-26	9.64	8.3*x*
				OP2014-03	8.48	7.3*x*
				OP2014-31	8.41	7.3*x*
				OP2014-21	8.16	7.0*x*
				OP2014-14	4.52	3.9*x*
			Rep 3	OP2014-01	8.53	7.4*x*
				OP2014-24	6.80	5.9*x*
			Rep 4	OP2014-22	9.37	8.1*x*
				OP2014-34	8.28	7.1*x*
			Rep 5	OP2014-19	8.16	7.0*x*

[z]Open-pollinated seedlings germinated from *H. syriacus* 'Aphrodite' and Bali™.
[y]Treatment with 125 µM oryzalin or 0.2% colchicine.
[x]Replicates representing lots of 15 seeds in a containers.
[w]Relative 2C genome sizes calculated in combined run with an internal standard (*Solanum lycopersicum* 'Stupicke'; 2C = 1.96 pg) on a flow cytometer.
[v]Ploidy estimate based on 1C*x* value calculated from average tetraploid genomes size from Table 3 (1C*x* = 4.64 pg / 4*x*). Ploidy estimate calculated as ploidy = holoploid genome size / 1.16 pg.

Table 6.6. Summary of FISH analysis across a ploidy series of *Hibiscus syriacus* L.

Taxa[z]	Accession[y]	Holoploid 2C genome size (pg)[x]	Ploidy[w]	5S rDNA signals (no.)[v]	45S rDNA signals (no.)[u]
Bali™	12-0023	4.61 ± 0.00	4*x*	2	4
'Pink Giant' x Bali™	H2013-078-03	5.65[t]	5*x*	2	4
'Pink Giant' x Lil' Kim™	H2013-084-16	6.03	5*x*	2	4
'Aphrodite' x 'Pink Giant'	H2013-017-11	5.81	5*x*	2	4
'Helene' x 'Pink Giant'	H2013-124-19	5.89	5*x*	2	5
'Pink Giant' x 'Red Heart'	H2013-085-02	6.07	5*x*	2	6
'Pink Giant' x Fiji™	H2013-129-08	5.64	5*x*	3	4
'Pink Giant' x 'Blushing Bride'	H2013-131-06	5.97	5*x*	3	4
'Pink Giant x 'Aphrodite'	H2013-077-05	5.64	5*x*	3	5
'Blue Bird' x 'Pink Giant'	H2013-044-03	5.60	5*x*	3	5
'Aphrodite' x 'Pink Giant'	H2013-017-21	5.70	5*x*	3	5
'Helene' x 'Pink Giant'	H2013-124-03	6.31	5*x*	3	6
'Diana' x 'Pink Giant'	H2013-049-01	5.71	5*x*	3	6
'Pink Giant'	11-0217	6.97 ± 0.07	6*x*	3	6
OP - Bali™ (oryzalin-treated seedling)	OP2014-19	8.88 ± 0.01	8*x*	4	8

[z]Taxa represent tetraploid Bali™, hexaploid 'Pink Giant', an octaploid oryzalin-treated, open-pollinated seedling of Bali™, and a range of pentaploid progeny recovered from reciprocal crosses with 'Pink Giant'.

[y]Accession number of parent taxa, hybrids, and experimental OP seedlings.

[x]Flow cytometry estimates based on leaf samples. Three leaf samples were evaluated for the 4*x*, 6*x*, and 8*x* cytotypes. A single leaf sample was evaluated for the 5*x* cytotypes.

[x]Estimated ploidy level based on three leaf samples analyzed by flow cytometry (mean ± SEM).

[w]Number of 5S rDNA signals determined using FISH.

[v]Number of 45 rDNS signals determined using FISH.

[t]Single leaf samples analyzed by flow cytometry among a large population of pentaploid seedlings to determine putative pentaploids for FISH.

Figures

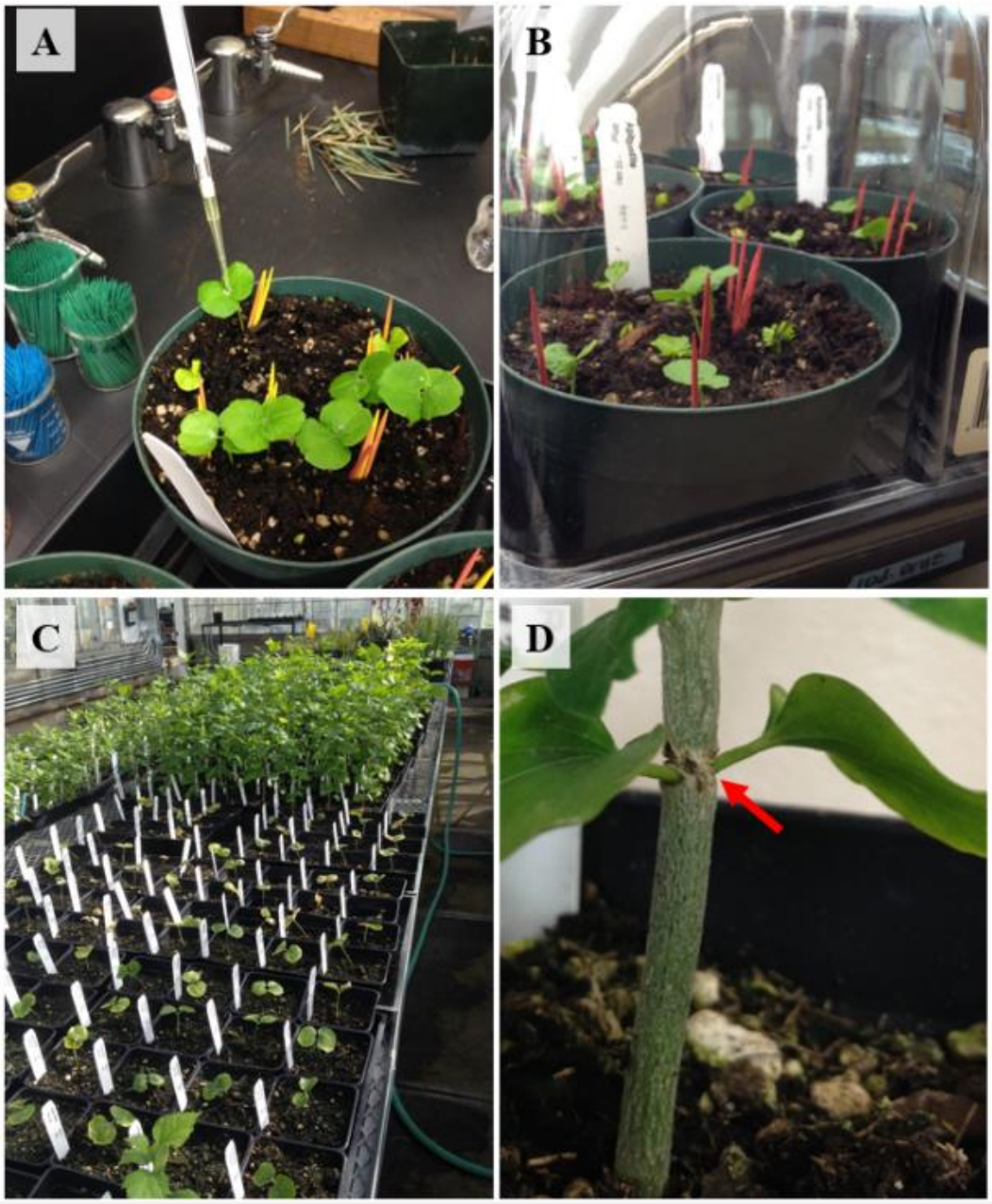

Fig. 6.1. Autopolyploid induction of *Hibiscus syriacus*. (A) Agar droplet application of oryzalin and colchicine to young meristems. (B) Droplets and seedlings maintained under humidity domes in under cool white fluorescent lights during treatments. (C) Plants potted into containers and grown in a glasshouse. (D) Necrosis at treatment site (indicated by red arrow) prompting cuttings of a subset of treated plants.

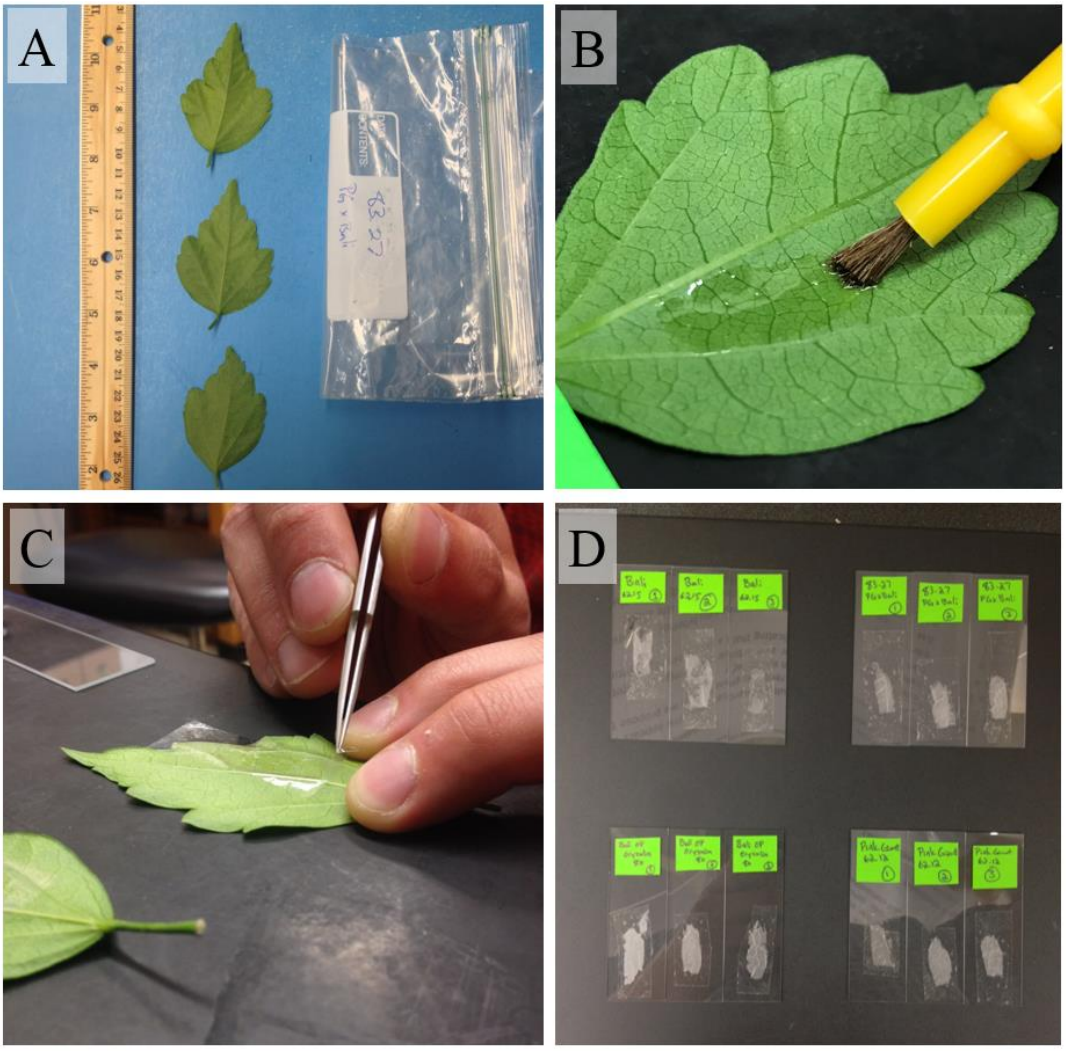

Fig. 6.2. Stomata cuticle peel on *Hibiscus syriacus* L. (A) Three random leaves of approximately the same size and age were collected fresh from each taxa investigated. (B) Clear fingernail polish was painted in a thin layer between major veins of the abaxial surface of each leaf and covered with a clear piece of packing tape. (C) After allowing the fingernail polish to dry, the packing tape was peeled from the leaf surface and placed on a clean microscope slide. (D) Three slides per taxa were labelled and viewed under a light microscope at ×200.

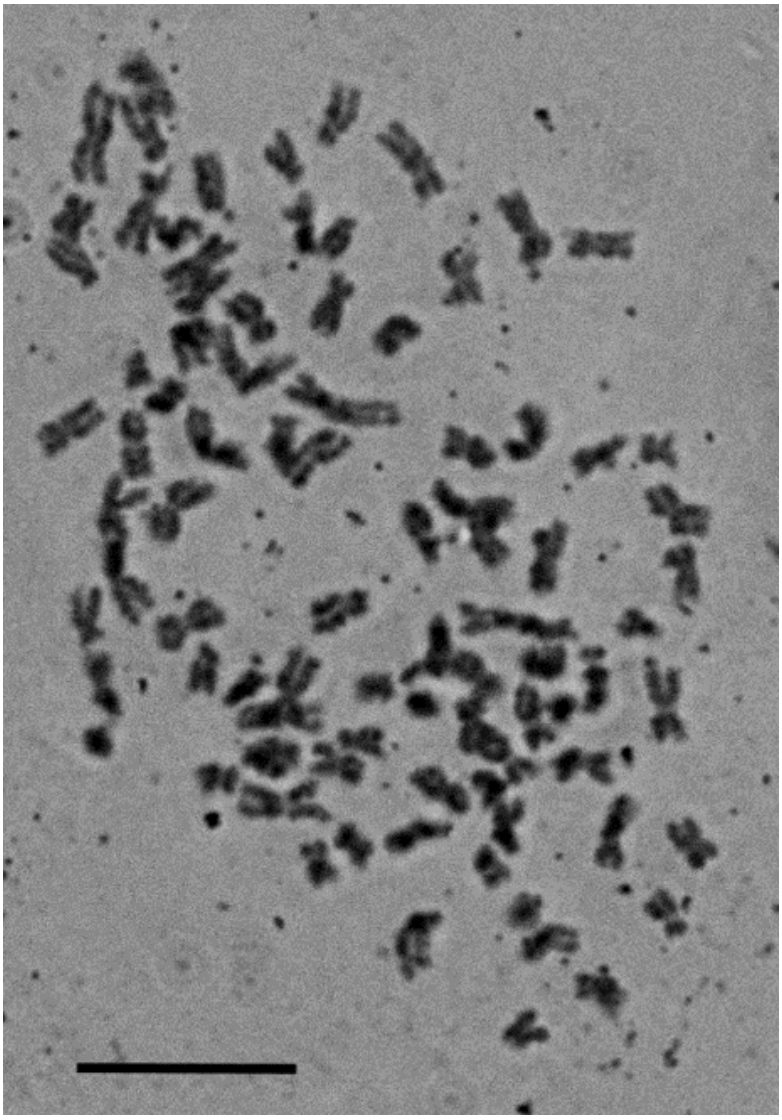

Fig. 6.3. Photomicrograph of metaphase chromosomes from root tip cells of *Hibiscus syriacus* ‘Diana’ ($2n = 4x = 80$). Viewed at ×1000. Scale bar at 10 μm.

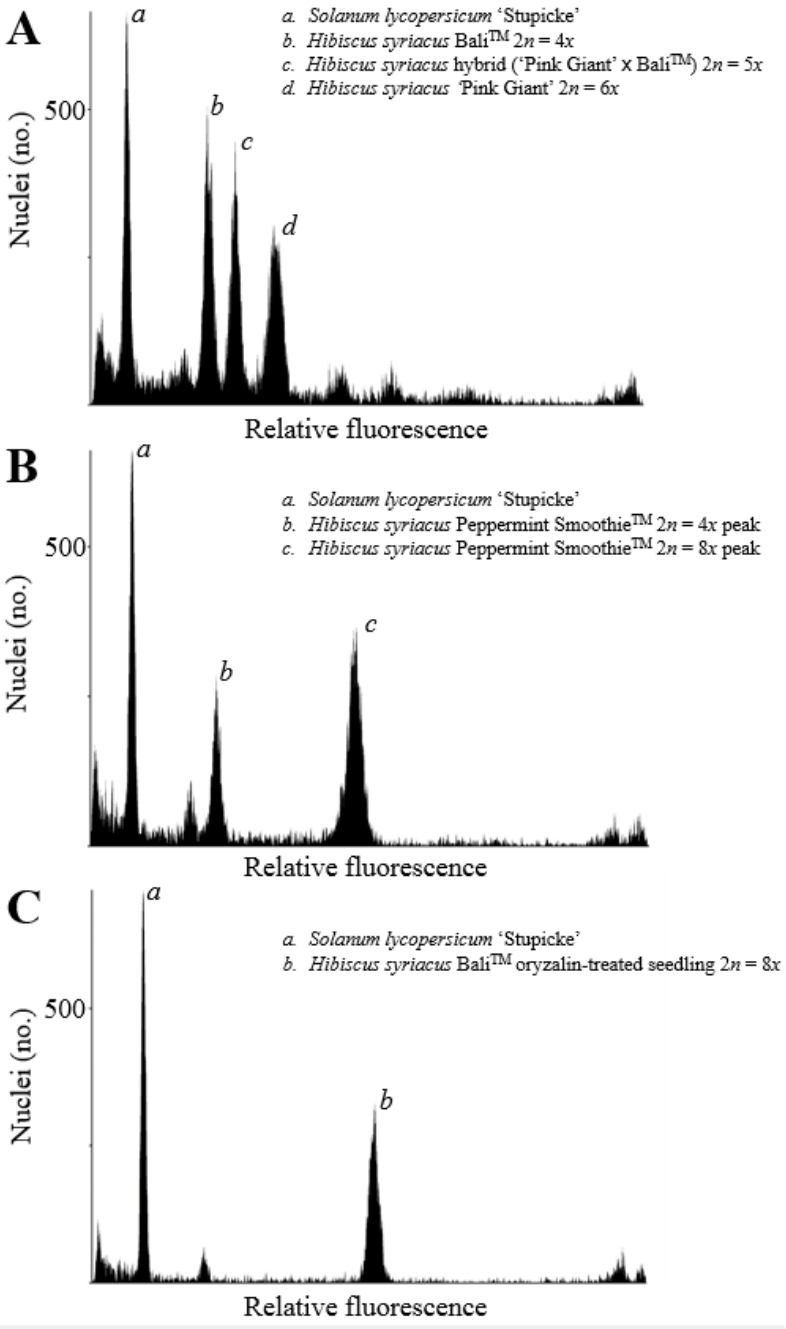

Fig. 6.4. Flow cytometry histograms of five *Hibiscus syriacus* cytotypes. Nuclei > 4,000 were used for each analysis and peaks with CV < 10. (A) Peaks represent the internal standard *Solanum lycopersicum* 'Stupicke' (2C = 1.96 pg), tetraploid *H. syriacus* Bali™ (2C = 4.64 pg), pentaploid hybrid H2013-078-01 *H. syriacus* 'Pink Giant x Bali™ (2C = 5.52 pg), and hexaploid *H. syriacus* 'Pink Giant' (2C = 6.84 pg). (B) Peaks represent the internal standard and cytochimera *H. syriacus* Peppermint Smoothie™ (tetraploid peak 2C = 4.72 pg; octaploid peak 2C = 9.23 pg). (C) Peaks represent the internal standard and the oryzalin-treated, open-pollinated seedling from *H. syriacus* Bali™ OP2014-19 (2C = 8.90 pg).

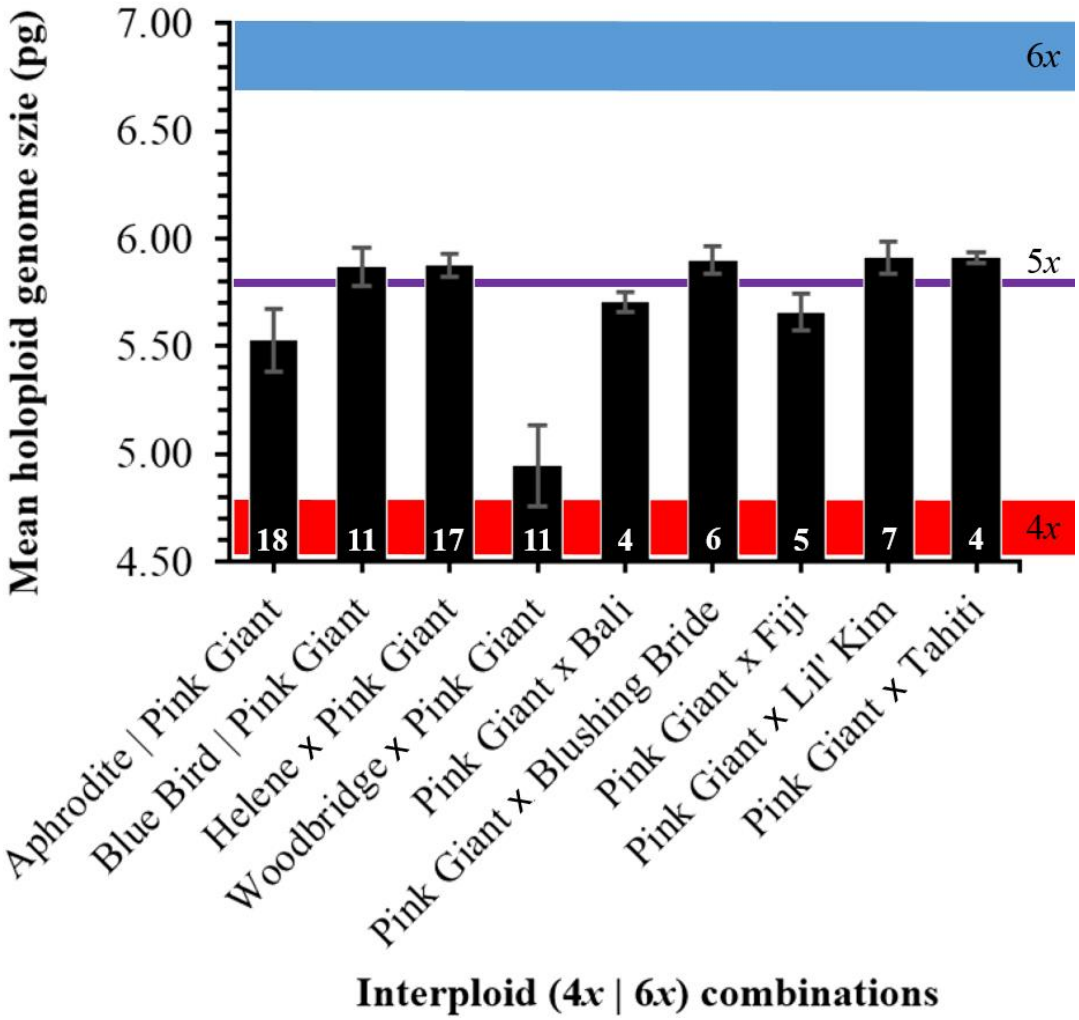

Fig. 6.5. Flow cytometry estimates of average holoploid 2C genome sizes in a subset interploid crosses between tetraploid *Hibiscus syriacus* cultivars and the hexaploid *H. syriacus* 'Pink Giant'. Reciprocal combinations indicated by "|" symbol while "x" represents a unidirectional cross. Black bars represent mean ± SEM for each reciprocal cross combination. White letters within black bars represent the number of seedlings measured for each cross. Red bar represents the range of tetraploid genome sizes found among cultivars of *H. syriacus* (Table 3). The blue bar represents the range of hexaploid genome sizes found among cultivars of *H. syriacus* (Table 3). The purple line represents the theoretical pentaploid genome size based on an average of mean 6*x* and 4*x* genome size ranges.

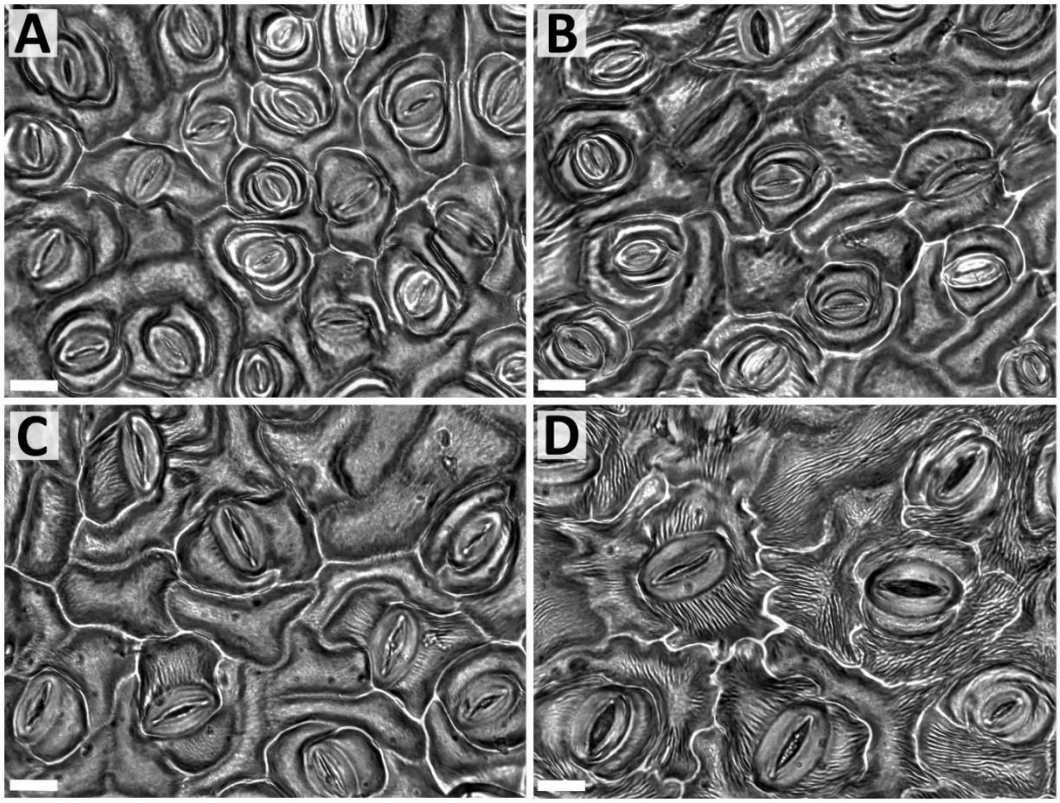

Fig. 6.6. Stomata anatomy from a ploidy series in *Hibiscus syriacus* L. Photomicrographs of cuticle peels viewed at ×630 magnification. Scale bar at 10 μm. (A) Tetraploid *H. syriacus* Bali™ 12-0023. (B) Pentaploid hybrid *H. syriacus* 'Pink Giant' x Bali™ H2013-078-01. (C) Hexaploid *H. syriacus* 'Pink Giant' 11-0217. (D) Octaploid, oryzalin-treated OP seedling from *H. syriacus* Bali™ OP2014-19.

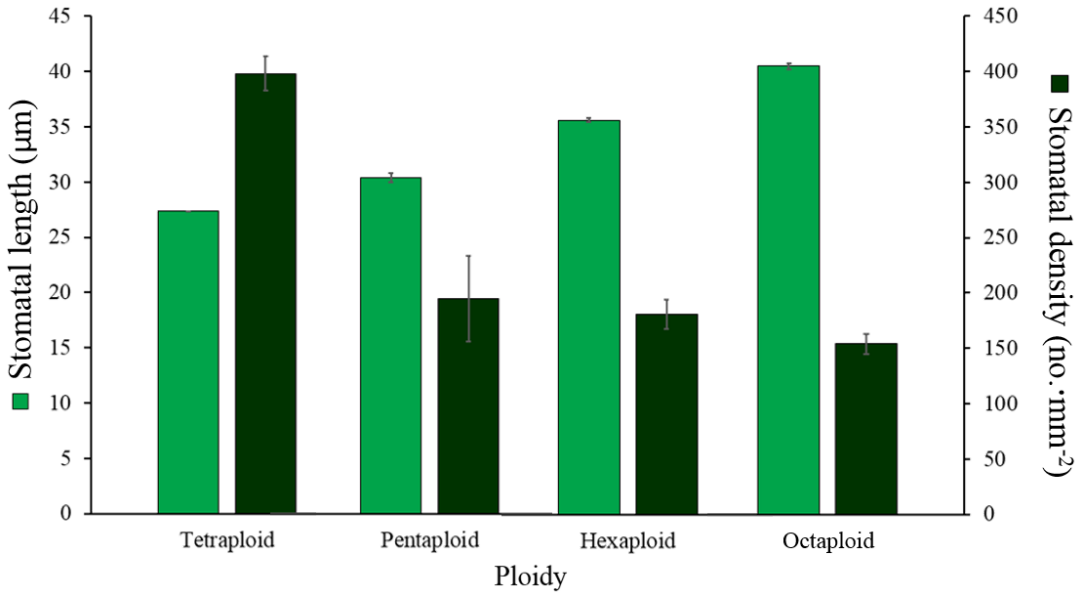

Fig. 6.7. Stomata length and density across a ploidy series in *Hibiscus syriacus* L. Ploidy series including tetraploid *H. syriacus* Bali™ (12-0023), pentaploid hybrid *H. syriacus* 'Pink Giant' x Bali™ (H2013-078-01), hexaploid *H. syriacus* 'Pink Giant' (11-0217), and octaploid, oryzalin-treated OP seedling from *H. syriacus* Bali™ (OP2014-19).

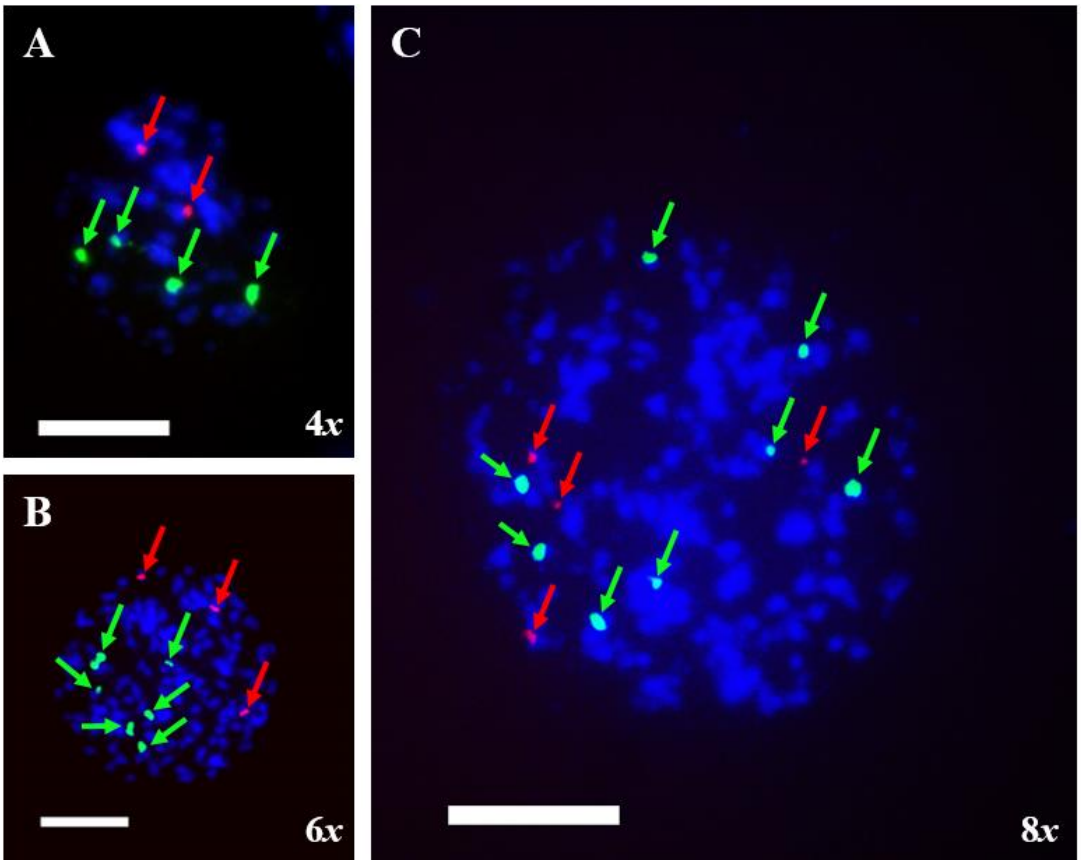

Fig. 6.8. Fluorescent in situ hybridization (FISH) analysis of metaphase, root tip chromosomes from in a ploidy series of *Hibiscus syriacus*. DAPI-stained chromosomes (blue) displaying variation in 5S (red) and 45S (green) rDNA loci. Scale bar represents 10 µm. (A) tetraploid *H. syriacus* Bali™ (two 5S signals + four 45 signals). (B) hexaploid *H. syriacus* 'Pink Giant' (three 5S signals + six 45S signals). (C) oryzalin-treated (125 µM for five days), octaploid seedling from *H. syriacus* Bali™ (four 5S signals + eight 45S signals).

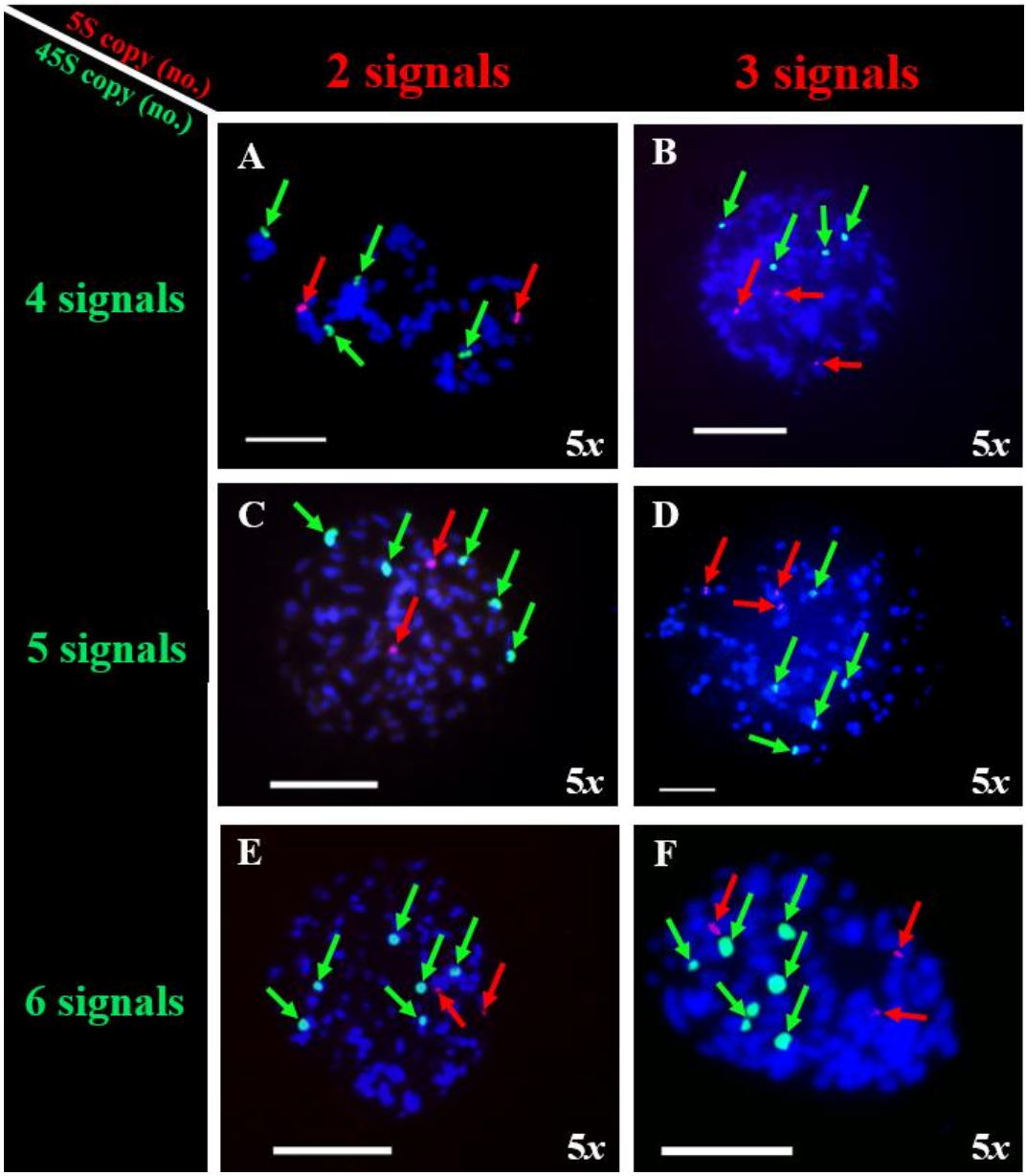

Fig. 6.9. Fluorescent in situ hybridization (FISH) analysis of metaphase, root tip chromosomes from a pentaploid seedlings of *Hibiscus syriacus*. DAPI-stained chromosomes (blue) displaying variation in 5S (red) and 45S (green) rDNA loci. Scale bar represents 10 µm. (A) H2013-078-03 'Pink Giant' x Bali™. (B) H2013-131-06 'Pink Giant' x 'Blushing Bride' (C) H2013-124-19 'Helene' x 'Pink Giant'. (D) H2013-017-21 'Aphrodite' x 'Pink Giant'. (E) H2013-085-02 'Pink Giant' x 'Red Heart'. (F) H2013-049-01 'Diana' x 'Pink Giant'.

CHAPTER 7: BREEDING FOR STERILITY IN ALTHEA (*Hibiscus syriacus* L.) – FERTILITY AMONG TETRAPLOID AND HEXAPLOID CULTIVARS, AND REDUCED FERTILITY IN PENTAPLOID HYBRIDS.

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CHAPTER 7: BREEDING FOR STERILITY IN ALTHEA (*Hibiscus syriacus* L.) – FERTILITY AMONG TETRAPLOID AND HEXAPLOID CULTIVARS, AND REDUCED FERTILITY IN PENTAPLOID HYBRIDS.

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Additional index words: ploidy, pollination, fertility, capsules, seeds.

Abstract. Althea (*Hibiscus syriacus*) is a popular shrub known for its vibrant summer blooms and winter hardiness. However, althea produces capsules with numerous, fertile seeds that germinate and cause a nuisance in production and the home landscape. Breeding for sterile forms of althea has long been a goal for *Hibiscus* breeders, yet many popular "sterile" cultivars have been reported as weedy. The purpose of this study was to evaluate female and male fertility of tetraploid and hexaploid cultivars, and to evaluate the female fertility of pentaploid progeny resulting from 4*x* x 6*x* and 6*x* x 4*x* crosses. Over 600 self-pollinations were performed on 21 cultivars, yet more than half of the taxa investigated were self-infertile. Only 24% of self-pollinations resulted in filled capsules for an overall rate of 4 seeds·pollination^{-1}. Significant differences were observed among taxa for seeds·capsule^{-1} ($P < 0.0001$) and seeds·pollination^{-1} ($P < 0.0001$). The most self-fertile taxa observed were White Chiffon® and Pink Chiffon®. A reduction in vigor was observed for the S_1 generation of most taxa. However, 'Woodbridge' produced vigorous seedling through the S_2 generation. Over 2000 cross-pollinations were also performed, resulting in over 15,000 seeds. Significant differences were found among taxa for identification of female fertility as seeds·capsule^{-1} ($P < 0.0001$) and

seeds·pollination^{-1} (P < 0.0001). In addition, significant differences were found among flower forms (single, semi-double, and double) for seeds·capsule^{-1} (P < 0.0001) and seeds·pollination^{-1} (P = 0.027). Double-flowered forms had reduced female fertility, which may indicate that breeding for increased petaloid stamens may result in a reduction in female fertility. Taxa previously reported to be sterile were found to be fertile, including 'Aphrodite', 'Diana', 'Helene' and 'Minerva'. Two hexaploids, 'Pink Giant' and Raspberry Smoothie™, had reduced female fertility compared to tetraploids. For male fertility, significant differences were found among taxa for seeds·capsule^{-1} (P < 0.0001) and seeds·pollination^{-1} (P = 0.035). However, no significant differences in male fertility were discovered among flower forms. Four taxa were found to have relatively high fertility with more than ten seeds·capsule^{-1} and seeds·pollination^{-1}, including Blue Satin®, Lil' Kim™, Bali™, and Tahiti™. Despite the significant differences among female and male fertility estimates for each taxa, individual cross combinations varied widely and fertility estimates are reported for all cross combinations. To test female fertility of pentaploids, pairwise comparisons were made between each taxa, and a control representing the average female fertility of tetraploid cultivars. Significant differences were found in pairwise comparisons between almost all pentaploid taxa and the control for seeds·capsule^{-1} and seeds·pollination^{-1}. Because seed production was minimal, average percent germination was calculated for each group (4*x* x 6*x* and 6*x* x 4*x*). No difference in percent germination was observed between the two groups (45% and 45%, respectively) but both were found to be significantly lower than the control group of open-pollinated tetraploid seed (89%). These reduced fertility estimates in

pentaploids will likely lead to new reduced fertility or sterile cultivars for the nursery industry. The combination of double flowers with pentaploid cytotypes will likely lead to completely sterile cultivars of althea.

Introduction

Weediness or invasive potential is a constant concern for ornamental shrubs and trees such as *Lantana* (Czarnecki et al., 2014), *Buddleja* (Tallent-Halsell and Watt, 2009), *Berberis* (Brand et al. 2012), *Ligustrum* (Fetouh et al., 2016) and *Acer* (Wangen and Webster, 2006). The elimination or reduction of seeds, especially in taxa with heavy seed production, has been a primary goal for ornamental plant breeders. Even in taxa that do not pose an immediate risk to native forests, weediness creates a constant maintenance issue in nursery production and in the home landscape. Sterile cultivars will likely save money, save time, and reduce the use of pesticides for commercial growers and home gardeners. In addition, for breeders, sterile cultivars that are clonally propagated could provide a built-in, genetic source of patent protection.

Fertility estimates of elite cultivars of ornamental plants can be beneficial for breeders to design future crosses. For breeders working on weedy and invasive species, fertility estimates have been shown to vary among genotypes. A fertility study on Japanese barberry (*Berberis thunbergii*), a plant considered invasive in ~30 states, identified sterile cultivars already available in the nursery trade (Brand et al. 2012). Of the 46 cultivars investigated, they found that seed production varied from no seed to more than 12,000 seeds per plant, while the number of seeds per fruit

ranged from 0.1 to 1.8 (Brand et al. 2012). However, mature plants that initially had low fertility were later evaluated and shown to be fertile, demonstrating that fertility can change from year to year and demonstrating the necessity for long-term fertility tests on cultivars (Brand et al., 2012).

Environmental groups often advocate for complete bans on weedy or invasive species, including all cultivars of proscribed species (Gagliardi and Brand, 2007). However, a majority of consumers (Kelley et al. 2006) and nursery and landscape professionals (Gagliardi and Brand, 2007) recognize that not all cultivars should be treated as invasive and banned. When surveyed about the best approach to reduce the sale of invasive plants, nursery and landscape professionals favored the creation of genetically altered sterile cultivars as one of their top choices (Gagliardi and Brand, 2007). Although natural mutation, induced mutations, and wide hybridization have been used to reduce or eliminate seed production, ploidy manipulation remains one of the more reliable tools for creating seedless or near-seedless clones, as illustrated by ornamental taxa such as *Hypericum* (Trueblood et al., 2010), *Buddleja* (Smith, 2010) and *Pyrus* (Phillips et al., 2015).

Fertility tests in plants with odd ploidy levels are useful for determining their rate of fertility, as seen in fertility tests of ten triploid accessions of the weedy species *Hypericum androsaemum* (Trueblood et al., 2010). Among the triploid accessions, Trueblood et al. (2010) found a significant reduction in male fertility and a complete elimination of viable seed production for nine out of the ten triploids. The triploids resulted in complete failure of nearly 171,000 potential fertilization events compared to the diploid controls (Trueblood et al., 2010). The focus of developing sterile

triploids is usually female fertility. However, in some cases, male sterility is also of great concern. Male sterility has been of great concern to breeders of *Lantana*, where the exotic ornamental *L. camara* outcrosses with the native *L. depressa* (Czarnecki et al., 2014). In a study of commercial cultivars and breeding lines of *L. camara*, triploids were found to be the most male sterile of the ploidy levels, followed by hexaploids, pentaploids, tetraploids, and diploids (Czarnecki et al., 2014). In addition, elite cultivars were found to vary widely in male fertility in studies of pollen stainability (Dehgan, 2006; Czarnecki et al., 2014).

Although triploids are often effective at eliminating fertility, odd ploidy levels are not always a guarantee of seedlessness. Higher level polyploids, such as pentaploids, have been found to be fertile or have reduced fertility in crops such as *Solanum* (Caruso et al., 2008), *Lantana* (Czarnecki et al., 2014) and *Vaccinium* (Laverty and Vorsa, 1991; Vorsa et al., 1987). In *Solanum*, Caruso et al. (2008) found that several pentaploid hybrids were female fertile when crossed with the tetraploid *S. tuberosum*. In addition, they found that the number of extra chromosomes in their aneuploid accessions had a significant effect on nearly all of their fertility parameters, including berry set, number of seeds per berry, and number of seeds per pollinated flower (Caruso et al., 2008). Their results agree with previous work in *Vaccinium* (Laverty and Vorsa, 1991) that showed a positive linear relationship between chromosome number and fertility estimates in aneuploids. One theory is that the higher the number of chromosomes, the more opportunity to produce gametes with compatible endosperm balance number (EBN) in resulting seed

(Caruso et al., 2008). For male sterility in *Lantana* polyploids, triploids had only 9.3% pollen stainability compared to 34.6% in pentaploids (Czarnecki et al., 2014).

Few guidelines exist to determine the acceptable rate of fertility in a cultivar of a potentially invasive plant. The only known example of a previously banned weedy ornamental plant allowing sterile or near-sterile cultivars is the case of *Buddleja* in Oregon. The Oregon Department of Agriculture (ODA) approved cultivars for sale in the state that have a 98% reduction in viable seed compared to industry standards (Contreras and McAninch, 2013). The threshold of two percent could provide a target for breeders seeking to create sterile forms of weedy or potentially invasive species.

Hibiscus syriacus is a clonally propagated ornamental shrub grown for its vibrant summer blooms beginning in late June and lasting until fall (Dirr, 2009). *Hibiscus syriacus* is one of the few hardy species in one of the most diversified genera in the Malvaceae (Bae et al., 2015). This versatile shrub is tolerant of numerous environmental conditions including a wide range of temperatures and soil conditions (Bae et al., 2015). In addition, *H. syriacus* can be a prolific seed producer, with part of its success due to herkogamous flowers. This type of pollination biology delays self-pollination by the spatial separation of stigma and anthers, but reflexing stigma provide a method of seed production when pollinators are scarce (Cheng-Jiang et al., 2009). After pollination, capsules produce numerous seeds that readily germinate and can become a nuisance in production and in the landscape (Dirr, 2009).

Hibiscus syriacus in the wild exists primarily as tetraploids ($2n = 4x = 80$) as reported by Skovsted (1941) and recently confirmed in a study to develop a draft

genome (Kim et al., 2017). Although no reports exist on higher ploidy levels in the wild, numerous reports describe polyploid induction experiments in *H. syriacus* (Eeckhaut et al., 2004; Egolf, 1970, 1981, 1986, 1988; Lee and Kim 1976; Shim et al., 1993; Van Huylenbroeck et al., 2000; Van Laere et al., 2006). Many of the cultivars produced from these studies have been reported as sterile or near-sterile including the U.S. National Arboretum releases 'Aphrodite', 'Diana', 'Minerva', and 'Helene'. However, no comprehensive study on fertility among cultivars of *H. syriacus* exists. In addition, no study exists evaluating the fertility of odd ploidy level *H. syriacus*. Therefore, the purpose of this study was to 1) evaluate the female and male fertility of tetraploid and hexaploid cultivars and 2) evaluate the female fertility of pentaploid progeny resulting from interploid hybridization in *H. syriacus*.

Methods and Materials

Plant materials. To test fertility of available plant materials, cultivars of *H. syriacus* were collected from botanical gardens, arboreta, and nurseries (Table 7.1). Both potted plants as well as cuttings were acquired. Plants were grown at Oregon State University and mature plants were grown at the Lewis Brown Horticulture Farm (Corvallis, OR). For each taxa, original cultivar and trademark names were maintained from each source. However, for *H. syriacus* and many ornamental taxa, usually one name becomes common in the nursery trade as the 'market name'. For simplicity, only market names (cultivar or trademark) will be used hereafter.

Intraploid cultivar crosses. Genome sizes and ploidy levels of each cultivar were determined using a combination of flow cytometry and root tip chromosome

counts (Chapter 5). From 2012 to 2014, a total of 204 combinations, representing both cross-pollinations and self-pollinations, were attempted among the tetraploid cultivars. Crosses were made in summer in a glasshouse kept free of pollinators with day/night temperatures of 25/20 °C and a 16-h photoperiod. Flowers were open for two days before stigmas reflexed in an effort to self-pollinate. Therefore, flowers were pollinated in the morning of their first flowering and stigmas were thoroughly covered with a dense layer of pollen. Fresh pollen was collected from flowers for the crosses on the day of pollination. Pollen of *H. syriacus* is large (108 to 169 μm) which prevents it from becoming airborne (Bae et al., 2015). It also produces numerous, long, sticky spines from its exine. There are 28 to 84 spines per grain with spine lengths of 8 to 25 μm, which cause the pollen to clump (Bae et al., 2015). Therefore, for pollination, clumps of pollen were placed on stigmas with forceps and forceps were sterilized in 70% ethanol between pollinations. When flowers were abundant, pollinations were performed directly using the monadelphous stamen of the male parent. Each cross was labelled with a jeweler's tag on which was recorded the parents and date. Each cross was observed daily for capsule development or flower abortion.

Tags were collected from aborted flowers throughout the summer and failed crosses were recorded in the fall. Viable capsules were monitored daily and capsules were collected two to three months post pollination, as the capsules began to turn yellow and sutures began to open. Data were collected on total number of pollinations, total number of filled capsules, and number of seeds per capsule. Preliminary germination tests from a variety of open-pollinated seed found that filled

seed yielded high germination irrespective of parent genotype. In addition, phytotoxicity from a pesticide spray one year, and an outbreak of *Pythium* the next, would have likely skewed germination data. Therefore, cross-compatibility and fertility estimates among cultivars were based on fruit and seed set. Non-stratified seeds from each cross were collected, cleaned, and sown into 1.3-L containers filled with growing medium (Metro-Mix; Sun Gro Horticulture, Agawam, MA) in seed lots of $\leq$ 30 seeds per container. Surviving seedlings were transplanted into 2.5-L containers filled with douglas-fir-based potting substrate during summer and grown under the conditions described above.

Interploid crosses. Interploid crosses were designed to create a seedling population of pentaploids. A total of 48 combinations, representing both cross-pollinations and self-pollinations, were attempted between tetraploid and hexaploid cultivars. Hexaploid taxa included were 'Pink Giant', Azurri Satin®, and Raspberry Smoothie™. Genome sizes and ploidy levels for hexaploid cultivars were determined using a combination of flow cytometry and root tip chromosome counts (Chapter 5). Pollinations, data collection, and seed germination were carried out as described above.

Pentaploid fertility testcrosses. In 2014, flow cytometry was performed on putative pentaploid seedlings created in 2012 and 2013, and cuttings were rooted for a subset of pentaploid seedlings. Cuttings were grown through the winter in a glasshouse under conditions described above. Fertility testcrosses were performed during 2015 and 2016. Each year, proven male-fertile cultivars were randomly selected to use as pollinizers. Each day, several randomly selected flowers were used

to pollinate all open flowers on pentaploid taxa. Tags were collected from aborted flowers throughout the summer and the number of failed crosses were recorded in fall. Viable capsules were monitored daily and capsules were collected two to three months post pollination, as the capsules began to yellow and sutures began to open. Data were collected on total number of pollinations, total number of filled capsules, and number of seed per capsule. Non-stratified seeds from each cross were collected, cleaned, and sown into 1.3-L containers filled with growing medium (Metro-Mix) in seed lots of $\leq$ 30 seeds per container. In addition, a positive control consisting of open-pollinated seed from proven fertile female taxa were sown. Three taxa were chosen: Blue Satin®, White Chiffon®, and 'Woodbridge' and ten seeds of each taxa were sown in five pots for a total of 150 seeds. Percent germination and number of albino progeny were counted for all treatments.

Statistical analyses. Due to unequal variances and sample sizes, all analyses of variance were conducted using a generalized mixed model analysis procedure (GLIMMIX) (SAS Studio; Cary, NC). For self-pollinations, taxa means were calculated for seeds·capsule^{-1} and seeds·pollination^{-1} using capsules and pollinations as a replicates, respectively. Flower form means were calculated for seeds·capsule^{-1} and seeds·pollination^{-1} using taxa means as replicates. Means comparisons were performed using the comparison lines test of GLIMMIX (α =0.05). For female fertility estimates of cross-pollinations, self-pollinations and interploid crosses were not included. The only exception was for female cross combinations using 'Aphrodite', 'Pink Giant' and Raspberry Smoothie™. These taxa were included to compare female fertility estimates with the tetraploid taxa. Taxa means were

calculated for seeds·capsule^{-1} and seeds·pollination^{-1} using means for each female cross combination as replicates. For flower form means, replicates were the genotype means. Means comparisons were performed using the comparison lines test of GLIMMIX (α = 0.05). For male fertility estimates of cross-pollination, self-pollinations and interploid crosses were not included. The only exception was for male cross combinations using 'Aphrodite', 'Pink Giant' and Raspberry Smoothie™. These taxa were included to compare male fertility estimates with the tetraploid taxa. Taxa means were calculated for seeds·capsule^{-1} and seeds·pollination^{-1} using means for each male cross combination as replicates. For flower form means, replicates were the genotype means. Means comparisons were performed using the comparison lines test of GLIMMIX (α = 0.05).

For seed set estimates of pentaploid progeny, means comparisons were performed for each taxa using a control based on a hypothetical average fertile tetraploid. The control seed·capsule^{-1} and control seed·pollination^{-1} were calculated from female fertility estimates of single and semi-double 4*x* x 4*x* crosses with a minimum of five pollinizers and a minimum of 30 total pollinations. Taxa included in the controls were Blue Satin®, 'Blue Bird', 'Buddha Belly', 'Diana', Lil' Kim™, 'Minerva', 'Red Heart', 'Woodbridge', Bali™, Blue Chiffon™, Fiji™, Tahiti™, and White Chiffon®. Taxa means were calculated for seeds·capsule^{-1} and seeds·pollination^{-1} using capsules and pollinations as a replicates, respectively. Means comparisons were performed using the comparison lines test of GLIMMIX (α = 0.05) with a Dunnett's adjustment for comparison to the controls. For germination estimates of pentaploid progeny, percent germination for each taxa were averaged

into an estimate for each interploid crossing group (4*x* x 6*x*) and (6*x* x 4*x*) and compared to percent germination of open-pollinated seed from three proven fertile taxa: Blue Satin®, White Chiffon®, and 'Woodbridge'. Means comparisons were performed using the comparison lines test of GLIMMIX ($\alpha = 0.05$) with a Dunnett's adjustment for comparison to the controls.

Results and Discussion

Self-pollinations. Self-pollinations were attempted in 21 cultivars of *H. syriacus*. Among all cultivars, a total of 631 self-pollinations were attempted resulting in 152 capsules and 2,356 seeds. This yielded filled capsules with 24% of pollinations and an overall seed set of 4 seeds·pollination^{-1}. Significant differences were found among individual taxa for seeds·capsule^{-1} ($P < 0.0001$) and seeds·pollination^{-1} ($P < 0.0001$). However, no significant differences were found between flower forms (single and semi-double flowers).

The most effective self-pollinations were observed in White Chiffon® and Pink Chiffon® with each resulting in 100% successful pollinations producing 25.8 ± 0.7 seeds·pollination^{-1} and 21.9 ± 0.08 seeds·pollination^{-1}, respectively (Table 7.2). Nearly half of the taxa investigated produced no capsules or seeds as a result of self-pollinations, including Bali™, 'Blushing Bride', 'Buddha Belly', China Chiffon™, 'Diana', Lavender Chiffon™, and 'Pink Giant' (Table 7.2). In addition, the only two single-flowering taxa without an eye spot ('Diana' and 'Buddha Belly') were found to be self-incompatible. Although many semi-double flowers of *H. syriacus* were self-fertile, most double-flowered taxa could not be self-pollinated due to lack of pollen.

Of the self-fertile taxa, eight were found to have fewer than 10 seeds·capsule^{-1} and 10 seeds·pollination^{-1} including 'Blue Bird', Blue Chiffon™, Blue Satin®, Fiji™, Hawaii™, 'Minerva', 'Red Heart', and Tahiti™ (Table 7.2).

After germination, an obvious reduction in vigor was observed in most self-pollinated (S_1) seedlings compared to cross-pollinated seedlings (J. Lattier – Personal Observation). This indicates that the development of inbred breeding lines in *H. syriacus* may be limited due to inbreeding depression. The only exceptions were observed in S_1 seedlings of White Chiffon®, Pink Chiffon®, 'Red Heart', and 'Woodbridge'. S_1 seedlings in White Chiffon® and 'Pink Chiffon' were vigorous and flowered in their first year from seed. However, they appeared more compact and with a larger number of petaloid stamens than their parents. Therefore, self-pollination of taxa with semi-double flowers may be an approach for breeders seeking to develop more compact double-flowered cultivars. In contrast, S_1 seedlings of 'Red Heart' and 'Woodbridge' grew tall and vigorous, with large, single flowers within their first year. In addition, 'Woodbridge' S_1 seedlings were self-pollinated, and the resulting S_2 seedlings also appear to be growing vigorously. The self-fertility of these cultivars could be due to a genetic effect, and may hint at their underlying levels of heterozygosity. However, pedigree information is scant on cultivars and further work will be necessary to discover the level of inbreeding possible in *H. syriacus*.

Female fertility. A total of 2342 cross-pollinations were attempted resulting in 973 capsules and 15,565 seeds. This yielded filled capsules from 38% of the pollinations and an overall seed set of 7 seeds·pollination^{-1} for all cross-pollinations. Significant differences were found among taxa for seeds·capsule^{-1} ($P < 0.0001$) and

seeds·pollination^{-1} (P < 0.0001). In addition, significant differences were found among flower forms (single, semi-double, and double) for seeds·capsule^{-1} (P < 0.0001) and seeds·pollination^{-1} (P = 0.027). Of the filled capsules, single flowers were observed to produce 17 ± 2 seeds·capsule^{-1} while double flowers produced only 9 ± 3 seeds·capsule^{-1} (Table 7.3). In addition, single and semi-double flowers were both found to produce more seeds·pollination^{-1} (8.6 ± 1.4 and 10.0 ± 1.7, respectively) than double flowers at 2.6 ± 2.0 seeds·pollination^{-1} (Table 7.3).

Cultivars previously reported to be sterile were found to be female fertile, including the USNA taxa, ‘Aphrodite’, ‘Diana’, ‘Helene’ and ‘Minerva’. Their rates of successful pollinations were 57%, 22%, 21%, and 55%, respectively (Table 7.3). Four hexaploid taxa previously identified by flow cytometry, including ‘Pink Giant’, Raspberry Smoothie™, Azurri Satin®, and a single “clone” of ‘Aphrodite’ (Chapter 5). Azurri Satin® was acquired near the end of the study. It had already produced numerous OP fruit but produced few new flowers for cross-pollinations (Table 7.3). However, OP fruit and seeds were collected, and germinated seedlings were recovered which exhibited pentaploid genome sizes (data not shown). The variation in genome size for “clones” of ‘Aphrodite’ were identified near the end of the pollination study. Therefore, the fertility estimates for ‘Aphrodite’ likely represent the combined fertility for tetraploid and hexaploid cytotypes of ‘Aphrodite’ (Table 7.3).

The remaining two hexaploids, ‘Pink Giant’ and Raspberry Smoothie™, represent a combined 371 pollinations (Table 7.3). The majority of crosses focused on combinations with ‘Pink Giant’, identified as a hexaploid at the beginning of the

study. Though fertile, none of the hexaploids yielded more than 4 ± 1 seeds·capsule^{-1}, compared to the most prolific tetraploid, White Chiffon®, at 26 ± 1 seeds·capsule^{-1} (Table 7.3). 'Pink Giant' yielded filled capsules with 11% of pollinations compared with 71% in Raspberry Smoothie™. In addition, 'Pink Giant' had some of the lowest fertility estimates among the cross-pollinations with 4 ± 0 seeds·capsule^{-1} and 0.4 ± 0.1 seeds·pollination^{-1} (Table 7.3). In contrast, Raspberry Smoothie™ had relatively high fertility estimates at 4 ± 1 seeds·capsule^{-1} and 2.7 ± 1.0 seeds·pollination^{-1}. Therefore, the relatively high female fertility of the double-flowered Raspberry Smoothie™ among hexaploids may represents a new opportunity for breeders to create double-flowered, pentaploid seedlings of *H. syriacus* (Table 7.3).

Flower form was found to have a significant effect on female fertility, as mentioned above. Of the taxa investigated, eight produced double flowers with all (or nearly all) of the stamens producing petals including 'Ardens', 'Blushing Bride', 'Collie Mullins', 'Lucy', Sugar Tip®. Raspberry Smoothie™, Strawberry Smoothie™, Blueberry Smoothie™, and Peppermint Smoothie™. Although these flowers produced only petaloid stamens, the majority produced normal or slightly contorted pistils. After many pollinations, several taxa proved to have no female fertility, including 'Ardens', 'Collie Mullins', and Sugar Tip®. Although Sugar Tip® did produce a single fruit and a single seed, no seedlings were recovered. Two taxa were classified as completely sterile due to the fact that stigmatic tissues were converted to petals on all flowers observed, including Peppermint Smoothie™ and Blueberry Smoothie™. Therefore, increasing the production of petaloid stamen may be a reliable mechanism for breeders to reduce fertility in *H. syriacus*.

However, several double-flowered taxa were observed to have normal pistils and produce viable offspring, including the hexaploid Raspberry Smoothie™ (mentioned above) and tetraploids ‘Blushing Bride’ and Strawberry Smoothie™ (Table 7.3). Of these fertile, double-flowered taxa, Raspberry Smoothie™ was the most prolific with capsules developed from 71% of pollinations, followed by ‘Blushing Bride’ with filled capsules for 42% of pollinations and Strawberry Smoothie with filled capsules for 27% of pollinations (Table 7.3).

Among the remaining tetraploid single flower and semi-double forms, all taxa were found to be female fertile with only six taxa producing more than ten per pollination, including Blue Satin®, ‘Buddha Belly’, Bali™, Blue Chiffon™, Pink Chiffon®, and White Chiffon®.

Male fertility. In addition to female fertility estimates, male fertility estimates were also calculated from cross-pollination results. For male fertility, significant differences were found among taxa for seeds·capsule^{-1} ($P < 0.0001$) and seeds·pollination^{-1} ($P = 0.035$). However, no significant differences in male fertility were discovered among flower forms (Table 7.4).

USNA taxa including ‘Aphrodite’, ‘Diana’, and ‘Minerva’ proved to be male-fertile with capsules developed from 30%, 47%, and 41% of pollinations, respectively (Table 7.4). Only one cross was attempted with ‘Helene’ as the plant was small and most flowers were used for female fertility tests. Further work will be necessary to determine if ‘Helene’ is male-fertile. Most double-flowered taxa produced only petaloid stamens with no pollen, and were therefore not included in male fertility estimates. However, ‘Blushing Bride’ proved a useful pollinizer in a few pollinations

with ‘Minerva’, yielding a single capsule containing six seeds (Table 7.4). Out of 500+ pollinations, the hexaploid taxa ‘Pink Giant’ developed capsules for 14% of pollinations (Table 7.4). In addition, ‘Pink Giant’ produced some of the lowest male fertility estimates at only 5.8 ± 0.6 seeds·capsule^{-1} and 1.6 ± 1.0 seeds·pollination^{-1} (Table 7.4). Of the remaining single and semi-double tetraploid plants, four taxa proved to have high fertility with more than ten seeds·capsule^{-1} and ten seeds·pollination^{-1}, including Blue Satin®, Lil’ Kim™, Bali™, and Tahiti™ (Table 7.4).

Individual crosses. Despite the significant differences found in female and male fertility for each taxa, individual cross combinations can vary widely. For instance, one of the most female fertile taxa, Bali™, produced a pollination rate of 94% when pollinated with ‘Diana’, with 16 capsules produced from 17 pollinations (Table 7.5). However, Bali™ produced a pollination rate of 0% when pollinated with Blue Chiffon™, with 0 capsules produce from 16 pollinations. Blue Chiffon™ had an overall pollination rate of 33% when used as a male in multiple cross combinations (Table 7.4). To aid future breeders of *H. syriacus*, cross-compatibility data has been reported on all attempted tetraploid crosses (Table 7.5). In addition, cross-compatibility data has been reported on all interploid (4*x* x 6*x*, 6*x* x 4*x*, and 6*x* x 6*x*) combinations (Table 7.6).

Pentaploid testcrosses. Pentaploid accessions resulting from crosses with hexaploid ‘Pink Giant’ grew slowly in their first two years and flowered sporadically. The majority of pentaploid accessions produced capsules and seeds from daily controlled crosses with randomly collected flowers from fertile male parents (Fig.

7.1A). Two novel floral phenotypes were observed among the pentaploids included in the fertility testcrosses. One accession (H2013-129-08) from the cross 'Pink Giant' x Fiji™ exhibited pink, bicolor flowers that never fully opened, and were reminiscent of a rose (Fig. 7.1B). Both parents produce flowers that fully open, and it is unclear whether the semi-closed flowers of H2013-129-08 was inherited from one of its parents or is a product of gigas effects from the odd ploidy level. However, no other pentaploid accession exhibited semi-closed flowers. The tetraploid male parent, Fiji™, is one of the only available taxa of *H. syriacus* to produce bi-color petals, with red-pink pigment present on the abaxial petal surface. One drawback to Fiji™ is that the pigment is most striking on the expanding flower bud, but less striking on the adaxial petal surface when the flower is fully open (Fig. 7.2B). Producing semi-closed, rose-like flowers in *H. syriacus* may be a novel way to enhance this ornamental characteristic when breeding with Fiji™ (Fig. 7.2A-B).

Another accession (H2013-131-06) produced large, petaloid male and female whorls, eliminating any possibility for fertility (Fig. 7.1C). Although pollinations could not be performed on this accession, observations were made on longevity of the flowers. Flowering was sporadic and inhibited by high levels of flower bud abortion, yet open flowers were observed to last up to two and a half weeks, compared to two days in a typical flower of *H. syriacus* (J. Lattier – Personal Observation). This striking flower longevity may indicate a longer bloom time as a byproduct of sterility in *H. syriacus*. Further work will be necessary to determine differences in flower duration and bloom time among accessions with different female fertility. All other pentaploid accessions produced large, single, pink flowers.

Of the 17 pentaploid accessions with 20+ attempted pollinations, four yielded a pollination rate of < 30% (filled capsule per pollination) including H2012-011-07 ('Bluebird' x 'Pink Giant') at 29%, H2013-078-03 ('Pink Giant' x Bali™) at 19%, H2013-084-21 ('Pink Giant' x Lil' Kim™) at 13%, and H2013-085-01 ('Pink Giant' x 'Red Heart') at 21% (Table 7.7). Significant differences were found in pairwise comparisons between pentaploid accessions and tetraploid controls for seeds·capsule^{-1} ($P < 0.0001$) and seeds·pollination^{-1} ($P < 0.0001$). Fertility estimates for most pentaploid accessions were significantly smaller than for a hypothetical control plant (based on average female fertility of tetraploid taxa) with 18 ± 2 seeds·capsule^{-1} and 9.9 ± 1.4 seeds·pollination^{-1} (Table 7.7). The most striking difference was observed as capsule began to dehisce, with many of the capsules producing no seed (Fig. 7.3A) or relatively few seed (Fig. 7.3B-C) compared with fertile tetraploids. Because of the low flower production and low seed set, percent germination estimates were obtained only from plants that produced at least 10 seeds. Significant differences in percent germination were discovered between pentaploids in the 4*x* x 6*x* treatment group, the 6*x* x 4*x* treatment group, and the open-pollinated seed from tetraploid controls ($P < 0.005$). An average percent germination of 45% was discovered for pentaploids from 4*x* x 6*x* combinations and 45% for pentaploids from the 6*x* x 4*x* combinations (Table 7.8). Therefore, an average pentaploid *H. syriacus* in the current study exhibited nearly half the percent germination of open-pollinated tetraploid seed at 89% (Table 7.8). In addition to reduced germination, and increased production of albino seedlings was observed in the pentaploid progeny (Fig. 7.3D). No albino seedlings were observed in the tetraploid controls.

In addition to fertility testcrosses on pentaploid taxa, the 2C holoploid genome size was analyzed for a subset of seedlings resulting from the testcrosses. From the analysis of single leaf samples from each seedling, the majority yielded tetraploid genome sizes. However, one seedling was found to be hexaploid (6.80 pg) from the cross H2013-124-13 (‘Helene’ x ‘Pink Giant’) x ‘Diana’. In addition, four seedlings were found to be heptaploid (7.21 pg to 7.60 pg) from the cross H2012-005-01 (‘Aphrodite’ x ‘Pink Giant’) x ‘Minerva’ and the cross H2013-124-13 (‘Helene’ x ‘Pink Giant’) x ‘Diana’. To our knowledge, this is the first report of heptaploid *H. syriacus* and these novel odd-ploidy seedlings may have reduced fertility in future testcrosses. In addition, these seedlings expand the ploidy series previously developed (Chapter 5) to five cytotypes for future research. Though only a single accession, the (near) decaploid (12.22 pg) seedling recovered from a previous polyploid induction experiment (Chapter 5) may also expand the ploidy series to six possible cytotypes: 4*x*, 5*x*, 6*x*, 7*x*, 8*x*, and 10*x*.

The combination of reduced capsule development and reduced seed·capsule^{-1} yield low seed·pollination^{-1} estimates for nearly all pentaploids illustrates their reduced fertility compared to fertile tetraploids. These reduced fertility estimates, combined with reduced germination rates, place many of the pentaploid taxa below the 2% relative fertility threshold outlined by the ODA (Contreras and McAninch. 2013). These taxa will likely lead to new reduced fertility or near-sterile cultivars of *H. syriacus* for the nursery industry. No obvious relationship in chromosome numbers (2C genome size) with fertility was found among the pentaploids tested, as

mentioned in other fertility studies with polyploids/aneuploids (Caruso, 2008; Laverty and Vorsa, 1991).

Future work will include a continuation of female and male fertility tests as pentaploid plants mature. Although less important, male fertility of pentaploids will also be evaluated by a combination of pollen staining and fertility testcrosses. Some crosses warrant repeating to produce more novel phenotypes, including interploid crosses with 'Blushing Bride' and Fiji™. Recently acquired hexaploids Azurri Satin® and Raspberry Smoothie™ will be used in further interploid combinations, especially the proven-fertile, double-flowered Raspberry Smoothie™. Future work will also include flow cytometry of the seedlings resulting from fertility testcrosses to develop new novel cytotypes of *H. syriacus*. These new seedlings could provide material to determine fertility rates among more interploid combinations. The combination of low fertility interploid hybrids with double flowers may lead to the production of new generations of novel, sterile *H. syriacus* for the nursery industry and home landscape.

Literature Cited

Bae, S.H., A. Younis, Y.-J. Hwang, and K.-B. Lim. 2015. Various pollen morphology in *Hibiscus syriacus*. Flower Res. J. 23:125-130.

Brand, M.H., J.M. Lehrer, and J.D. Lubell. 2012. Fecundity of Japanese barberry (*Berberis thunbergii*) cultivars and their ability to invade a deciduous woodland. Invasive Plant Science and Management 5:464-476.

Caruso, I., L. Castaldi, G. Caruso, L. Frusciante, and D. Carputo. 2008. Breeding potential of *Solanum tuberosum-S. commersonii* pentaploid hybrids: fertility studies and tuber evaluation. Euphytica 164:357-363.

Contreras, R.N. and G. McAninch. 2013. Back from the ban: new *Buddleja* cultivars receive exemption under ODA amendment. Digger (October):33-36.

Cheng-Jiang, R., Q. Pei, J.A. Teixeira da Silva, and Q.-X. Zhang. 2009. Movement herkogamy in *Kosteletzkya virginica*: effect on reproductive success and contribution to pollen receipt and reproductive assurance. Acta Ecologica Sinica 29:98-103.

Czarnecki II, D.M., A.J. Hershberger, C.D. Robacker, D.G. Clark, and Zhanao Deng. 2014. Ploidy levels and pollen stainability of *Lantana camara* cultivars and breeding lines. HortScience 49:1271-1276.

Dehgan, B. 2006. Reproductive biology and invasive potential of *Lantana camara* cultivars.

U.S. Department of Agriculture Research, Education & Economics. Inf. Syst. 7 July 2017. <http://www.reeis.usda.gov/web/crisprojectpages/0191420-reproductivebiology-and-invasive-potential-of-lantanacamara-cultivars.html>.

Dirr, M.A. 2009. Manual of woody landscape plants: their identification, ornamental characteristics, culture, propagation and uses. 6th ed. Stipes Publishing, Champaign, IL.

Eeckhaut, T.G.R., J.M. Van Huylenbroeck, J. De Riek, and E. Van Bockstaele. 2004. Interspecific hybridization between *Hibiscus syriacus* L. and *Hibiscus paramutabilis* Bailey. Acta Hort. (ISHS) 630:85-90.

Egolf, D.R. 1970. *Hibiscus syriacus* 'Diana', a new cultivar. Baileya. 17:75-78.

Egolf, D.R. 1970. 'Helene' rose of sharon (althea). HortScience 16:226-227.

Egolf, D.R. 1986. 'Minerva' rose of sharon (althea). HortScience 21:1463-1464.

Egolf, D.R. 1988. 'Aphrodite' rose of sharon (althea). HortScience 23:223.

Gagliardi, J.A. and M.H. Brand. 2007. Connecticut nursery and landscape industry preferences for solutions to the sale and use of invasive plants. HortTechnology 17:39-45.

Kelley, K.M., J.R. Conklin, J.C. Sellmer, and R.M. Bates. 2006. Invasive plant species: results of a consumer awareness, knowledge, and expectations survey conducted in Pennsylvania. J. Environ. Hort. 24:53-58.

Kim, Y.-M., S. Kim, N. Koo, A.-Y. Shin, S.-I. Yeom, E. Seo, S.-J. Park, W.-H. Kang, M.-S. Kim, J. Park, I. Jang, P.-G. Kim, I. Byeon, M.-S. Kim, J. Choi, G. Ko, J. Hwang, T.-J. Yang, S.-B. Choi, J.M. Lee, K.-B. Lim, J. Lee, I.-Y. Choi, B.-S. Park, S.-Y. Kwon, D. Choi, and R.W. Kim. 2017. Genome analysis of

Hibiscus syriacus insights of polyploidization and indeterminate flowering in woody plants. DNA Res. 24:71-80.

Lee, S.K. and C.S. Kim. 1976. Studies on artificial polyploidy forest trees XIII: Some morphological and physiological characteristics of colchitetraploid *Hibiscus syriacus* L. J. Korean For. Soc. 32:73-86.

Phillips, W.D., T.G. Ranney, D.H. Touchell, and T.A. Eaker. 2015. Developing non-invasive callery pears: fertility and reproductive biology of triploid flowering pears (*Pyrus* sp.). HortScience 51:968-971.

Shim, K.K., K.H. Kim, and Y.M. Ha. 1993. Characteristics of triploid cultivars 'Diana' and 'Helene' in *Hibiscus syriacus* L. J. Korean Soc. Hort. Sci. 34:54-67.

Smith, W.A. 2010. Development of novel breeding stock in *Buddleja davidii* through mutation breeding and haploid induction. Ph. D. dissertation. CT: University of Connecticut. 163 p.

Skovsted, A. 1941. Chromosome numbers in the Malvaceae II. Comptes Rendus des Travaux du Laboratoire Carlsberg Série Physiologique 23:195-242.

Tallent-Halsell, N.G., and M.S. Watt. The invasive *Buddleja davidii* (Butterfly Bush). Bot. Rev. 75:292-325.

Van Huylenbroeck, J.M., J. De Riek, and M. De Loose. 2000. Genetic relationships among *Hibiscus syriacus*, *Hibiscus sinosyriacus* and *Hibiscus paramutabilis* revealed by AFLP, morphology and ploidy analysis. Genet. Res. Crop Evol. 47:335-343.

Van Laere, K., J. Van Huylenbroeck, and E. Van Bockstaele. 2006. Breeding strategies for genetic variability within *Hibiscus syriacus*. Acta Hort. (ISHS) 714:75-81.

Wangen, S.R., and C.R. Webster. 2006. Potential for multiple lag phases during biotic invasions: reconstructing an invasion of the exotic tree *Acer platanoides*. J. Appl. Ecol. 43:258-268.

Tables

Table 7.1. Source material for *Hibiscus syriacus* L. breeding at Oregon State University.

Cultivar[z]	Trade Name[y]	Accession[x]	Source[w]
'American Irene Scott'	Sugar Tip®	12-0019	Bailey Nurseries
'Antong Two'	Lil' Kim™	12-0021	Bailey Nurseries
'Aphrodite'		13-0054	Monrovia
		11-0215	Bailey Nurseries
'Ardens'		13-0050	Blue Heron
'Blue Bird'		11-0219	Monrovia
		13-0057	Monrovia
'Blushing Bride'		13-0048	Blue Heron
		13-0059	Monrovia
'Bricutts'	China Chiffon™	13-0060	Monrovia
'Buddha Belly'		14-0128	Yamaguchi Nursery
'Collie Mullins'		13-0061	Monrovia
'Diana'		13-0062	Monrovia
		11-0211	Bailey Nurseries
'DS01BS'	Blueberry Smoothie™	14-0092	Greenleaf Nursery
'DS02SS'	Strawberry Smoothie™	14-0091	Greenleaf Nursery
'DS03RS'	Raspberry Smoothie™	14-0094	Greenleaf Nursery
'DS04PS'	Peppermint Smoothie™	14-0093	Greenleaf Nursery
'DVPazurri'	Azurri Satin®	13-0055	Monrovia
		14-0188	Spring Meadow
		16-0015	Forestfarm
'Floru'	Violet Satin®	13-0118	JC Raulston Arboretum
		13-0119	JC Raulston Arboretum
'Helene'		13-0063	Monrovia
		13-0116	JC Raulston Arboretum
		13-0117	JC Raulston Arboretum
'JWNfour'	Pink Chiffon®	13-0067	Monrovia
'Lucy'		11-0216	Bailey Nurseries
'Marina'	Blue Satin®	13-0094	JC Raulston Arboretum
		11-0210	Bailey's Nursery
'Mathilde'	Blush Satin®	13-0058	Monrovia
'Mineru'	First Editions® Tahiti™	12-0024	Bailey Nurseries
		13-0098	Bailey Nurseries
'Minerva'		13-0051	Blue Heron
		13-0066	Monrovia
		11-0213	Bailey Nurseries
'Minfren'	First Editions® Bali™	12-0023	Bailey Nurseries
'Minrosa'	Rose Satin®	13-0068	Monrovia
		13-0068	Monrovia

Table 7.1 (continued). Source material for *Hibiscus syriacus* L. breeding at Oregon State University.

Cultivar[z]	Trade Name[y]	Accession[x]	Source[w]
'Minspot'	First Editions® Fiji™	12-0022	Bailey Nurseries
'Minsygrbl1'	First Editions® Hawaii™	12-0020	Bailey Nurseries
		13-0096	Bailey Nurseries
'Notwoodone'	Lavender Chiffon™	13-0046	Blue Heron
		13-0064	Monrovia
'Notwoodthree'	Blue Chiffon™	13-0056	Monrovia
		11-0218	Blue Heron
'Notwoodtwo'	White Chiffon®	13-0044	Blue Heron
'Pink Giant'		11-0217	Bailey Nurseries
'Red Heart'		13-0049	Blue Heron
'Woodbridge'		11-0214	Bailey Nurseries
		13-0047	Blue Heron

[z]Cultivar name.

[y]Trademark name.

[x]Accession number in research collection at the Ornamental Plant Breeding Lab, Oregon State University, Corvallis, OR.

[w]Container plant collected from the following sources: Bailey Nurseries, Yamhill, OR; Blue Heron Farm, Corvallis, OR; Forestfarm Nursery, Williams, OR; Greenleaf Nursery, Grants Pass, OR; JC Raulston Arboretum, Raleigh, NC; Monrovia, Dayton, OR; Spring Meadow Nursery, Grand Haven, MI; Yamaguchi Plantsman Nursery, Gifu, Japan.

Table 7.2. Self-pollination fertility estimates for cultivars of *Hibiscus syriacus* L.

Flower form[z]	Taxon	Ploidy[y]	Pollinations (no.)	Capsules (no.)	Pollination (%)[x]	Seeds (no.)	Seeds·capsule^{-1} (mean ± SE)[w]	Seeds·pollination^{-1} (mean ± SE)[v]
Single							13 ± 1 A	4.0 ± 1.6 A
	'Aphrodite'	4*x*	70	22	31	374	17 ± 2 C	5.3 ± 1.1 C
	'Blue Bird'	4*x*	39	12	31	89	7 ± 1 D	2.3 ± 0.6 CD
	Blue Satin®	4*x*	39	22	56	186	8 ± 1 D	4.8 ± 0.8 C
	'Buddha Belly'	4*x*	27	0	0	0	0	0.0 ± 0.0 D
	'Diana'	4*x*	139	0	0	0	0	0.0 ± 0.0 D
	Hawaii™	4*x*	4	4	100	9	2 ± 1 D	2.3 ± 0.9 CD
	Lil' Kim™	4*x*	47	28	60	549	20 ± 1 BC	11.4 ± 1.6 B
	'Minerva'	4*x*	2	2	100	10	5	5.0
	'Pink Giant'	6*x*	29	0	0	0	0	0.0 ± 0.0 D
	'Red Heart'	4*x*	32	3	9	16	5 ± 1 D	0.5 ± 0.3 D
	'Woodbridge'	4*x*	30	20	67	411	21 ± 2 BC	13.7 ± 2.2 B
Semi-double							14 ± 4 A	6.1 ± 3.9 A
	Bali™	4*x*	23	0	0	0	0	0.0 ± 0.0 D
	Blue Chiffon™	4*x*	49	3	6	13	4 ± 0 D	0.3 ± 0.2 D
	China Chiffon™	4*x*	11	0	0	0	0	0.0 ± 0.0 D
	Fiji™	4*x*	37	6	16	24	4 ± 1 D	0.6 ± 0.3 D
	Lavender Chiffon™	4*x*	8	0	0	0	0	0.0 ± 0.0 D
	Pink Chiffon®	4*x*	20	20	100	439	22 ± 1 AB	21.9 ± 0.8 A
	Tahiti™	4*x*	10	1	10	5	5	0.5 ± 0.5 D
	White Chiffon®	4*x*	9	9	100	232	26 ± 1 A	25.8 ± 0.7 A
Double							0	0.0
	'Blushing Bride'	4*x*	6	0	0	0	0	0.0 ± 0.0 D

[z]Flower forms based on number of petaloid stamen.

[y]Ploidy estimates based on flow cytometry and root tip squashes (Chapter 5). Ploidy series later discovered for 'Aphrodite' and 'Minerva' were used in crosses. MV = missing value.

[x]Percent pollination calculated per genotype as: [total filled capsules / total pollinations] × 100.

[w]Average seeds per capsule calculated among flower form (within boxes) and among genotypes. Among flower forms, replicates are the genotype means. Letters separating least squares (LS) means based on comparison lines test of the generalized linear mixed model procedure (GLIMMIX). Among genotypes, replicates are capsules. Letters separating LS means based on comparison lines test of GLIMMIX.

[v]Average seeds per pollination calculated among flower form (within boxes) and among genotypes. Among flower forms, replicates are the genotype means. Letters separating LS means based on comparison lines test of GLIMMIX. Among genotypes, replicates are pollinations. Letters separating LS means based on comparison lines test of GLIMMIX.

Table 7.3. Female fertility estimates for flower forms and female genotypes of *Hibiscus syriacus* L.

Flower form[z]	♀ parent	Ploidy[y]	♂ parents (no.)	Pollinations (no.)	Capsules (no.)	Pollination (%)[x]	Seeds (no.)	Seeds·capsule^{-1} (mean ± SE)[w]	Seeds·pollination^{-1} (mean ± SE)[v]
Single								17 ± 2 A	8.6 ± 1.4 A
	‘Aphrodite’	4*x*	9	121	69	57	1406	20 ± 1 BC	11.6 ± 2.5 B-D
	Azurri Satin®	6*x*	2	2	0	0	0	0	0.0
	Blue Satin®	4*x*	11	107	75	70	1626	22 ± 1 B	14.1 ± 2.6 A-C
	‘Blue Bird’	4*x*	14	240	109	45	1798	17 ± 1 D-F	8.3 ± 2.2 C-F
	‘Buddha Belly’	4*x*	6	42	34	81	752	22 ± 1 B	19.2 ± 3.1 A
	‘Diana’	4*x*	15	395	87	22	1302	15 ± 1 D-F	5.6 ± 1.5 D-F
	Hawaii™	4*x*	4	17	13	76	123	9 ± 1 F-H	7.8 ± 2.6 C-F
	‘Helene’	MV	6	28	6	21	103	17 ± 7 B-F	5.7 ± 3.2 D-F
	Lil’ Kim™	4*x*	10	74	28	38	440	16 ± 1 D-F	5.3 ± 2.4 D-F
	‘Minerva’	4*x*	10	40	22	55	311	14 ± 2 EF	6.3 ± 2.5 D-F
	‘Pink Giant’	6*x*	18	350	40	11	149	4 ± 0 H	0.4 ± 0.1 F
	‘Red Heart’	4*x*	11	173	62	36	1720	28 ± 2 A	9.8 ± 2.9 C-E
	‘Woodbridge’	4*x*	11	115	45	39	805	18 ± 1 B-E	8.5 ± 2.5 C-F
Semi-double								15 ± 2 AB	10.0 ± 1.7 A
	Bali™	4*x*	9	110	78	71	1443	19 ± 1 B-D	11.5 ± 2.6 B-D
	Blue Chiffon™	4*x*	9	127	76	60	1410	19 ± 1 B-D	16.7 ± 3.0 AB
	China Chiffon™	4*x*	2	11	6	55	59	10 ± 3 F-H	8.1
	Fiji™	4*x*	10	47	25	53	383	15 ± 1 D-F	9.3 ± 1.9 C-E
	Lavender Chiffon™	4*x*	3	36	19	53	133	7 ± 1 GH	3.9 ± 0.8 D-F
	Pink Chiffon®	4*x*	4	39	33	85	456	14 ± 1 E-G	10.4 ± 3.4 B-E
	Tahiti™	4*x*	8	39	6	15	29	5 ± 1 H	1.1 ± 0.5 F
	White Chiffon®	4*x*	6	40	25	63	690	28 ± 1 A	18.6 ± 4.8 AB
Double								9 ± 3 B	2.6 ± 2.0 B
	‘Ardens’	4*x*	3	12	0	0	0	0	0.0 ± 0.0 F
	‘Blushing Bride’	4*x*	6	43	18	42	302	17 ± 2 C-F	8.8 ± 2.5 C-F
	‘Collie Mullins’	4*x*	2	18	0	0	0	0 ± 0	0.0
	‘Lucy’	4*x*	12	60	5	8	30	6 ± 2 GH	0.4 ± 0.3 F

Table 7.3 (continued). Female fertility estimates for flower forms and female genotypes of *Hibiscus syriacus* L.

Flower form[z]	♀ parent	Ploidy[y]	♂ parents (no.)	Pollinations (no.)	Capsules (no.)	Pollination (%)[x]	Seeds (no.)	Seeds·capsule^{-1} (mean ± SE)[w]	Seeds·pollination^{-1} (mean ± SE)[v]
	Raspberry Smoothie™	6*x*	5	21	15	71	61	4 ± 1 H	2.7 ± 1.0 EF
	Strawberry Smoothie™	4*x*	3	15	4	27	33	8 ± 2 F-H	3.5 ± 1.9 D-F
	Sugar Tip®	4*x*	2	22	1	5	1	1	0.0

[z]Flower forms based on number of petaloid stamen.

[y]Ploidy estimates based on flow cytometry and root tip squashes (Chapter 5). Ploidy series later discovered for 'Aphrodite' and 'Minerva' were used in crosses. MV = missing value.

[x]Percent pollination calculated per genotype as: [total filled capsules / total pollinations] × 100.

[w]Average seeds per capsule calculated among flower form (within boxes) and among genotypes. Self-pollinations were not included. Interploid crosses were not included, except for 'Aphrodite', 'Pink Giant', and Raspberry Smoothie™. Among flower forms, replicates are the genotype means. Letters separating least squares (LS) means based on comparison lines test of the generalized linear mixed model procedure (GLIMMIX). Among genotypes, replicates are capsules. Letters separating LS means based on comparison lines test of GLIMMIX.

[v]Average seeds per pollination calculated among flower form (within boxes) and among genotypes. Self-pollinations were not included. Interploid crosses were not included, except for 'Aphrodite', 'Pink Giant', and Raspberry Smoothie™. Among flower forms, replicates are the genotype means. Letters separating LS means based on comparison lines test of GLIMMIX. Among genotypes, replicates are means for each female cross combination. Letters separating LS means based on comparison lines test of GLIMMIX.

Table 7.4. Male fertility estimates for cultivars of *Hibiscus syriacus* L.

Flower form[z]	♂ parent	Ploidy[y]	♀ parents (no.)	Pollinations (no.)	Capsules (no.)	Pollination (%)[x]	Seeds (no.)	Seeds·capsule^{-1} (mean ± SE)[w]	Seeds·pollination^{-1} (mean ± SE)[v]
Single								16.0 ± 1.5 A	7.4 ± 0.9 A
	'Aphrodite'	4*x*	11	122	36	30	590	16.7 ± 1.5 B-D	6.9 ± 2.5 A-C
	Blue Satin®	4*x*	15	133	57	43	1203	20.7 ± 1.5 A	10.2 ± 2.5 AB
	'Blue Bird'	4*x*	14	124	43	35	763	17.2 ± 1.8 A-D	7.3 ± 2.7 AB
	'Buddha Belly'	4*x*	7	49	28	57	612	21.9 ± 1.3 A	7.3 ± 3.7 A-C
	'Diana'	4*x*	19	380	177	47	3534	20.0 ± 0.8 AB	9.2 ± 2.1 AB
	Hawaii™	4*x*	2	26	3	12	25	8.3 ± 3.4 DE	na
	'Helene'	MV	1	17	0	0	0	0.0	na
	Lil' Kim™	4*x*	13	114	77	68	1319	17.1 ± 0.7 B-D	12.7 ± 1.9 A
	'Minerva'	4*x*	15	96	39	41	516	13.4 ± 2.0 DE	5.6 ± 1.3 BC
	'Pink Giant'	6*x*	23	527	72	14	415	5.8 ± 0.6 E	1.6 ± 1.0 C
	'Red Heart'	4*x*	14	208	50	24	813	16.3 ± 1.5 CD	5.8 ± 2.5 BC
	'Woodbridge'	4*x*	13	119	50	42	939	18.9 ± 1.3 A-D	6.9 ± 2.5 A-C
Semi-double								17.1 ± 2.3 A	9.4 ± 0.7 A
	Bali™	4*x*	11	109	64	59	1174	18.3 ± 1.1 A-D	10.7 ± 2.5 AB
	Blue Chiffon™	4*x*	13	148	49	33	959	19.6 ± 1.3 A-C	7.3 ± 2.2 AB
	China Chiffon™	4*x*	2	27	5	19	128	25.6 ± 1.0 A	na
	Fiji™	4*x*	13	109	65	60	1040	16.0 ± 1.2 DE	9.0 ± 2.1 AB
	Pink Chiffon®	4*x*	1	3	3	100	16	5.3 ± 0.9 E	na
	Tahiti™	4*x*	10	75	50	67	917	18.3 ± 1.1 A-D	11.1 ± 2.1 AB
	White Chiffon®	4*x*	12	110	49	45	801	16.3 ± 1.0 CD	8.9 ± 2.5 AB
Double								na	na
	'Blushing Bride'	4*x*	1	4	1	25	6	6.0	na

[z]Flower forms based on number of petaloid stamen.

[y]Ploidy estimates based on flow cytometry and root tip squashes (Chapter 5). Ploidy series later discovered for 'Aphrodite' and 'Minerva' were used in crosses. MV = missing value.

[x]Percent pollination calculated per genotype as: [total filled capsules / total pollinations] × 100.

[w]Average seeds per capsule calculated among flower form (within boxes) and among genotypes. Self-pollinations were not included. Interploid crosses were not included, except for 'Aphrodite', 'Pink Giant', and Raspberry Smoothie™. Among flower forms, replicates are the genotype means. Letters separating least squares (LS) means based on comparison lines test of the generalized linear mixed model procedure (GLIMMIX). Among genotypes, replicates are capsules. Letters separating LS means based on comparison lines test of GLIMMIX.

[v]Average seeds per pollination calculated among flower form (within boxes) and among genotypes. Self-pollinations were not included. Interploid crosses were not included, except for 'Aphrodite', 'Pink Giant', and Raspberry Smoothie™. Among flower forms, replicates are the genotype means. Letters separating LS means based on comparison lines test of GLIMMIX. Among genotypes, replicates are means for each female cross combination. Letters separating LS means based on comparison lines test of GLIMMIX.

Table 7.5. Fertility estimates for each tetraploid cross combination of *Hibiscus syriacus* L.

♀ parent	♂ parent	Pollinations (no.)	Capsules (no.)	Pollination (%)[z]	Seeds (no.)	Seeds·capsule^{-1} (mean ± SE)[y]	Seeds·pollination^{-1} [x]
'Aphrodite'	Bali™	23	10	43	235	24 ± 3	10
	Blue Satin®	8	4	50	117	29 ± 4	15
	'Blue Bird'	5	4	80	143	36 ± 1	29
	'Diana'	31	22	71	470	21 ± 2	15
	Fiji™	21	14	67	195	14 ± 2	9
	Lil' Kim™	5	1	20	18	18	4
	'Minerva'	7	5	71	70	14 ± 6	10
	'Red Heart'	14	7	50	137	20 ± 4	10
	'Woodbridge'	7	2	29	21	11 ± 3	3
'Ardens'	'Buddha Belly'	9	0	0	0	0 ± 0	0
	'Diana'	2	0	0	0	0 ± 0	0
	White Chiffon®	1	0	0	0	0 ± 0	0
Bali™	'Aphrodite'	18	13	72	252	19 ± 2	14
	Blue Chiffon™	16	0	0	0	0 ± 0	0
	'Blue Bird'	20	18	90	329	18 ± 2	16
	'Diana'	17	16	94	325	20 ± 2	19
	Fiji™	4	2	50	4	2 ± 1	1
	Lil' Kim™	5	3	60	50	17 ± 4	10
	'Minerva'	5	3	60	24	8 ± 4	5
	Tahiti™	5	4	80	101	25 ± 2	20
	'Woodbridge'	20	19	95	358	19 ± 2	18
Blue Chiffon™	'Aphrodite'	5	5	100	133	27 ± 3	27
	Bali™	22	6	27	66	11 ± 2	3
	'Diana'	43	21	49	250	12 ± 1	6
	Fiji™	8	6	75	138	23 ± 2	17
	Lil' Kim™	5	5	100	86	17 ± 4	17
	'Red Heart'	22	17	77	339	20 ± 2	15
	Tahiti™	15	9	60	191	21 ± 2	13
	White Chiffon®	1	1	100	21	21	21
	'Woodbridge'	6	6	100	186	31 ± 2	31

Table 7.5 (continued). Fertility estimates for each tetraploid cross combination of *Hibiscus syriacus* L.

♀ parent	♂ parent	Pollinations (no.)	Capsules (no.)	Pollination (%)[z]	Seeds (no.)	Seeds·capsule^{-1} (mean ± SE)[y]	Seeds·pollination^{-1} [x]
Blue Satin®	'Aphrodite'	7	4	57	15	4 ± 1	2
	Bali™	11	9	82	202	22 ± 3	18
	'Blue Bird'	5	3	60	75	MV	15
	'Buddha Belly'	16	16	100	419	26 ± 1	26
	'Diana'	20	16	80	347	23 ± 2	17
	Lil' Kim™	7	6	86	145	24 ± 3	21
	'Minerva'	10	4	40	52	13 ± 6	5
	'Red Heart'	9	6	67	162	27 ± 2	18
	Tahiti™	2	2	100	17	9 ± 1	9
	White Chiffon®	8	8	100	187	23 ± 2	23
	'Woodbridge'	12	1	8	5	MV	0
'Blue Bird'	'Aphrodite'	6	2	33	21	MV	4
	Bali™	23	22	96	328	15 ± 2	14
	Blue Chiffon™	10	10	100	256	26 ± 1	26
	Blue Satin®	15	12	80	183	15 ± 3	12
	'Buddha Belly'	5	1	20	26	26	5
	China Chiffon™	6	4	67	103	26 ± 1	17
	'Diana'	47	3	6	10	3 ± 1	0
	Fiji™	26	13	50	124	10 ± 1	5
	Hawaii™	19	1	5	6	MV	0
	Lil' Kim™	29	21	72	381	18 ± 1	13
	'Minerva'	13	3	23	30	MV	2
	'Red Heart'	16	0	0	0	0 ± 0	0
	Tahiti™	19	16	84	329	21 ± 2	17
	'Woodbridge'	6	1	17	1	MV	0
'Blushing Bride'	'Aphrodite'	5	2	40	27	14 ± 7	5
	Blue Chiffon™	9	6	67	101	17 ± 4	11
	'Diana'	18	3	17	30	10 ± 4	2
	Fiji™	5	3	60	46	15 ± 1	9
	'Minerva'	6	4	67	98	25 ± 2	16

Table 7.5 (continued). Fertility estimates for each tetraploid cross combination of *Hibiscus syriacus* L.

♀ parent	♂ parent	Pollinations (no.)	Capsules (no.)	Pollination (%)[z]	Seeds (no.)	Seeds·capsule^{-1} (mean ± SE)[y]	Seeds·pollination^{-1} [x]
'Buddha Belly'	Blue Chiffon™	7	4	57	92	23 ± 6	13
	Blue Satin®	2	2	100	51	26 ± 7	26
	'Diana'	28	24	86	524	22 ± 1	19
	Lil' Kim™	2	2	100	25	13 ± 2	13
	'Red Heart'	1	1	100	31	MV	31
	White Chiffon®	2	1	50	29	MV	15
China Chiffon™	Blue Chiffon™	3	3	100	42	14 ± 5	14
	'Blue Bird'	8	3	38	17	6 ± 0	2
'Collie Mullins'	Blue Chiffon™	13	0	0	0	MV	0
	'Diana'	5	0	0	0	MV	0
'Diana'	'Aphrodite'	49	5	10	74	15 ± 3	2
	Bali™	5	4	80	37	9 ± 2	7
	Blue Chiffon™	39	4	10	104	26 ± 2	3
	Blue Satin®	28	11	39	198	18 ± 2	7
	'Blue Bird'	29	0	0	0	MV	0
	'Buddha Belly'	10	8	80	138	17 ± 2	14
	China Chiffon™	21	1	5	25	MV	1
	Fiji™	6	5	83	120	24 ± 4	20
	'Helene'	17	0	0	0	MV	0
	Lil' Kim™	27	17	63	227	13 ± 1	8
	'Minerva'	12	5	42	70	20 ± 14	6
	'Red Heart'	101	13	13	115	9 ± 2	1
	Tahiti™	11	8	73	135	17 ± 2	12
	White Chiffon®	19	2	11	25	MV	1
	'Woodbridge'	21	4	19	34	8 ± 3	2
Fiji™	'Aphrodite'	1	1	100	16	MV	16
	Bali™	4	2	50	48	24 ± 3	12
	Blue Chiffon™	7	6	86	59	10 ± 3	8
	'Blue Bird'	7	2	29	16	8 ± 5	2
	'Diana'	5	0	0	0	MV	0
	Hawaii™	7	2	29	19	MV	3

Table 7.5 (continued). Fertility estimates for each tetraploid cross combination of *Hibiscus syriacus* L.

♀ parent	♂ parent	Pollinations (no.)	Capsules (no.)	Pollination (%)[z]	Seeds (no.)	Seeds·capsule^{-1} (mean ± SE)[y]	Seeds·pollination^{-1} [x]
Fiji™	Lil' Kim™	3	2	67	37	MV	12
	'Minerva'	1	1	100	10	MV	10
	White Chiffon®	6	5	83	110	22 ± 2	18
	'Woodbridge'	6	4	67	68	17 ± 2	11
Hawaii™	Fiji™	4	4	100	45	11 ± 3	11
	'Minerva'	7	6	86	51	9 ± 1	7
	'Red Heart'	4	1	25	3	MV	1
	Tahiti™	2	2	100	24	12 ± 4	12
'Helene'	Blue Satin®	2	1	50	37	MV	19
	'Blue Bird'	2	0	0	0	0 ± 0	0
	'Diana'	18	3	17	25	8 ± 2	1
	'Minerva'	3	1	33	38	MV	13
	'Red Heart'	2	1	50	3	MV	2
	'Woodbridge'	1	0	0	0	MV	0
Lavender Chiffon™	'Diana'	25	13	52	96	7 ± 1	4
	Pink Chiffon®	3	3	100	16	5 ± 1	5
	White Chiffon®	8	3	38	21	7 ± 2	3
Lil' Kim™	Bali™	4	2	50	30	MV	8
	Blue Chiffon™	10	4	40	61	15 ± 3	6
	'Blue Bird'	6	2	33	23	12 ± 4	4
	'Buddha Belly'	3	0	0	0	MV	0
	'Diana'	1	1	100	17	17	17
	Fiji™	1	0	0	0	MV	0
	'Red Heart'	3	0	0	0	MV	0
	Tahiti™	6	2	33	25	13 ± 9	4
	White Chiffon®	28	12	43	204	17 ± 1	7
	'Woodbridge'	12	5	42	80	16 ± 1	7
'Lucy'	'Aphrodite'	2	0	0	0	MV	0
	Bali™	5	0	0	0	MV	0
	Blue Chiffon™	1	0	0	0	MV	0
	Blue Satin®	14	0	0	0	MV	0
	'Blue Bird'	5	0	0	0	MV	0
	'Buddha Belly'	1	0	0	0	MV	0

Table 7.5 (continued). Fertility estimates for each tetraploid cross combination of *Hibiscus syriacus* L.

♀ parent	♂ parent	Pollinations (no.)	Capsules (no.)	Pollination (%)[z]	Seeds (no.)	Seeds·capsule^{-1} (mean ± SE)[y]	Seeds·pollination^{-1} [x]
'Lucy'	'Diana'	7	3	43	18	6 ± 2	3
	Fiji™	5	0	0	0	MV	0
	Lil' Kim™	4	0	0	0	MV	0
	'Minerva'	4	1	25	1	MV	0
	'Red Heart'	5	1	20	11	MV	2
	White Chiffon®	7	0	0	0	MV	0
'Minerva'	Bali™	2	0	0	0	MV	0
	Blue Satin®	2	1	50	17	MV	9
	'Blue Bird'	3	3	100	20	7 ± 1	7
	'Blushing Bride'	4	1	25	6	MV	2
	'Diana'	7	2	29	14	MV	2
	Fiji™	8	8	100	185	23 ± 4	23
	Lil' Kim™	3	3	100	48	16 ± 1	16
	'Red Heart'	4	1	25	2	MV	1
	Tahiti™	4	3	75	19	6 ± 1	5
	'Woodbridge'	3	0	0	0	MV	0
Pink Chiffon®	Blue Satin®	10	6	60	44	7 ± 1	4
	'Buddha Belly'	5	3	60	29	10 ± 3	6
	'Diana'	13	13	100	250	19 ± 1	19
	White Chiffon®	11	11	100	133	12 ± 1	12
'Red Heart'	'Aphrodite'	9	2	22	36	MV	4
	Bali™	4	3	75	77	26 ± 1	19
	Blue Chiffon™	17	12	71	244	20 ± 3	14
	Blue Satin®	8	6	75	167	27 ± 4	21
	'Blue Bird'	15	1	7	5	MV	0
	'Diana'	76	30	39	997	33 ± 2	13
	Lil' Kim™	1	1	100	27	MV	27
	'Minerva'	9	0	0	0	MV	0
	Tahiti™	7	0	0	0	MV	0
	White Chiffon®	7	1	14	2	MV	0
	'Woodbridge'	20	6	30	165	28 ± 4	8
Strawberry Smoothie™	Blue Satin®	8	0	0	0	MV	0
	'Minerva'	5	3	60	20	7 ± 2	4

Table 7.5 (continued). Fertility estimates for each tetraploid cross combination of *Hibiscus syriacus* L.

♀ parent	♂ parent	Pollinations (no.)	Capsules (no.)	Pollination (%)[z]	Seeds (no.)	Seeds·capsule^{-1} (mean ± SE)[y]	Seeds·pollination^{-1} [x]
Strawberry Smoothie™	'Woodbridge'	2	1	50	13	13	7
Sugar Tip®	Blue Chiffon™	13	0	0	0	MV	0
	Blue Satin®	9	1	11	1	MV	0
Tahiti™	'Aphrodite'	3	1	33	8	MV	3
	Blue Chiffon™	3	0	0	0	MV	0
	Blue Satin®	8	0	0	0	MV	0
	'Blue Bird'	10	2	20	3	MV	0
	Fiji™	3	1	33	7	MV	2
	'Minerva'	2	0	0	0	MV	0
	'Red Heart'	7	1	14	3	MV	0
	'Woodbridge'	3	1	33	8	MV	3
White Chiffon®	Blue Satin®	10	9	90	267	30 ± 1	27
	'Blue Bird'	5	5	100	132	26 ± 3	26
	'Diana'	2	2	100	63	MV	32
	Fiji™	10	5	50	135	27 ± 1	14
	Lil' Kim™	7	4	57	93	23 ± 3	13
	'Red Heart'	6	0	0	0	MV	0
'Woodbridge'	'Aphrodite'	17	1	6	8	MV	0
	Bali™	6	6	100	151	25 ± 2	25
	Blue Satin®	8	4	50	121	38 ± 1	15
	'Blue Bird'	4	0	0	0	MV	0
	'Diana'	15	5	33	98	20 ± 3	7
	Fiji™	8	4	50	41	10 ± 4	5
	Lil' Kim™	16	12	75	182	15 ± 2	11
	'Minerva'	11	3	27	52	MV	5
	'Red Heart'	14	1	7	7	MV	1
	Tahiti™	4	4	100	76	19 ± 5	19
	White Chiffon®	12	5	42	69	14 ± 4	6

[z]Percent pollination calculated as: [capsules / pollinations] × 100.

[y]Average seeds per capsule calculated using capsules for each combination as replicates. MV = missing value.

[x]Total seed per pollination calculated as: [total seed / total pollination] × 100. MV = missing value.

Table 7.6. Fertility estimates for each interploid (tetraploid and hexaploid) cross combination of *Hibiscus syriacus* L.

Ploidy[z]	♀ parent	♂ parent	Pollinations (no.)	Capsules (no.)	Pollination (%)[y]	Seed (no.)	Seeds·capsule^{-1} (mean ± SE)[x]	Seeds·pollination^{-1} [w]
4*x* × 6*x*								
	'Aphrodite'	'Pink Giant'	10	7	70	31	4 ± 1	3
	'Ardens'	'Pink Giant'	45	0	0	0	MV	0
	Bali™	'Pink Giant'	11	3	27	6	2 ± 1	1
	Blue Chiffon™	'Pink Giant'	19	0	0	0	MV	0
	Blue Satin®	'Pink Giant'	25	15	60	87	6 ± 1	3
	'Blue Bird'	'Pink Giant'	17	5	29	27	5 ± 2	2
	'Blushing Bride'	'Pink Giant'	45	12	27	81	7 ± 1	2
	'Buddha Belly'	'Pink Giant'	6	0	0	0	MV	0
	China Chiffon™	'Pink Giant'	27	0	0	0	MV	0
	'Collie Mullins'	'Pink Giant'	19	0	0	0	MV	0
	'Diana'	'Pink Giant'	42	2	5	7	MV	0
	Fiji™	'Pink Giant'	22	4	18	8	2 ± 1	0
	'Helene'	'Pink Giant'	1	1	100	23	MV	23
	Lil' Kim™	'Pink Giant'	48	4	8	28	7 ± 2	1
	'Lucy'	'Pink Giant'	17	0	0	0	MV	0
	'Minerva'	'Pink Giant'	9	3	33	4	1 ± 0	0
	Pink Chiffon®	'Pink Giant'	71	5	7	36	7 ± 2	1
	'Red Heart'	'Pink Giant'	14	0	0	0	MV	0
	Strawberry Smoothie™	'Pink Giant'	14	0	0	0	MV	0
	Sugar Tip®	'Pink Giant'	7	0	0	0	MV	0
	Tahiti™	'Pink Giant'	4	1	25	1	MV	0
	White Chiffon®	'Pink Giant'	42	3	7	41	14 ± 6	1
	'Woodbridge'	'Pink Giant'	31	7	23	35	5 ± 3	1
6*x* × 4*x*								
	Azurri Satin®	Blue Satin®	1	0	0	0	MV	0
	Azurri Satin®	'Minerva'	1	0	0	0	MV	0
	'Pink Giant'	Bali™	25	3	12	8	3 ± 1	0
	'Pink Giant'	Blue Chiffon™	32	4	13	11	3 ± 1	0
	'Pink Giant'	Blue Satin®	33	2	6	2	MV	0
	'Pink Giant'	'Blue Bird'	5	3	60	5	2 ± 1	1
	'Pink Giant'	'Blushing Bride'	33	3	9	21	7 ± 2	1
	'Pink Giant'	China Chiffon™	3	0	0	0	MV	0

Table 7.6 (continued). Fertility estimates for each interploid (tetraploid and hexaploid) cross combination of *Hibiscus syriacus* L.

Ploidy[z]	♀ parent	♂ parent	Pollinations (no.)	Capsules (no.)	Pollination (%)[y]	Seed (no.)	Seeds·capsule^{-1} (mean ± SE)[x]	Seeds·pollination^{-1} [w]
6*x* × 4*x*								
(cont.)	'Pink Giant'	'Diana'	28	2	7	13	MV	0
	'Pink Giant'	Fiji™	18	4	22	22	6 ± 2	1
	'Pink Giant'	Hawaii™	7	0	0	0	MV	0
	'Pink Giant'	Lavender Chiffon™	3	1	33	1	MV	0
	'Pink Giant'	Lil' Kim™	32	8	25	34	4 ± 1	1
	'Pink Giant'	'Minerva'	5	0	0	0	MV	0
	'Pink Giant'	Pink Chiffon®	1	0	0	0	MV	0
	'Pink Giant'	'Red Heart'	13	5	38	16	3 ± 0	1
	'Pink Giant'	Tahiti™	15	4	27	11	3 ± 1	1
	'Pink Giant'	White Chiffon®	80	0	0	0	MV	0
	'Pink Giant'	'Woodbridge'	7	0	0	0	MV	0
	Raspberry Smoothie™	Blue Satin®	4	3	75	5	2 ± 1	1
	Raspberry Smoothie™	'Blue Bird'	3	0	0	0	MV	0
	Raspberry Smoothie™	'Diana'	5	3	60	27	9 ± 6	5
	Raspberry Smoothie™	'Minerva'	4	4	100	18	5 ± 1	5
	Raspberry Smoothie™	'Woodbridge'	5	5	100	11	2 ± 1	2

[z]Interploid combinations included 4*x* x 6*x* and 6*x* x 4*x*.

[y]Percent pollination calculated as: [capsules / pollinations] × 100.

[x]Average seeds per capsule calculated using capsules for each combination as replicates. MV = missing value.

[w]Total seed per pollination calculated as: [total seed / total pollination] × 100. MV = missing value.

Table 7.7. Pollination, capsule, and seed estimates from testcrosses on progeny resulting from combinations of tetraploid and hexaploid *Hibiscus syriacus* L.

Treatment[z]	♀ pedigree[y]	♀ 5*x* accession no.	♀ 2C genome size (pg)[x]	Pollinations (no.)	Capsules (no.)	Pollination (%)[w]	Seeds (no.)	Seeds·capsule^{-1} (mean ± SE)[v]	Seeds.pollination-1 (mean ± SE)u
4*x* x 6*x*									
	'Aphrodite' x PG	H2012-005-01	6.4	51	34	67	188	5.5 ± 0.4 A	3.7 ± 0.5 A
		H2013-017-21	5.7	12	9	75	27	3.0 ± 0.7 A	2.3 ± 0.7 A
		H2013-017-16	6.1	58	23	40	40	1.7 ± 0.3 A	0.7 ± 0.2 A
	'Bluebird' x PG	H2012-011-02	5.8	2	0	0	0	MV	MV
		H2012-011-04	6.3	3	2	67	0	MV	0.0 ± 0.0 A
		H2012-011-07	6.1	24	7	29	4	0.6 ± 0.3 A	0.2 ± 0.1 A
	'Diana' x PG	H2013-049-01	5.7	3	1	33	0	MV	0.0 ± 0.0 A
	'Helene' x PG	H2013-124-13	6.1	81	77	95	552	7.1 ± 1.7 A	6.8 ± 1.8 A
		H2013-124-19	5.9	14	9	64	5	0.6 ± 0.4 A	0.4 ± 0.3 A
		H2013-124-03	6.3	29	27	93	64	2.3 ± 0.4 A	2.2 ± 0.4 A
	'Woodbridge' x PG	H2012-041-01	6.8	1	1	100	1	MV	MV
6*x* x 4*x*									
	PG x 'Aphrodite'	H2013-077-05	5.6	38	23	61	289	12.6 ± 1.4 A	7.6 ± 1.3 B
	PG x Bali™	H2013-078-03	5.7	21	4	19	5	1.3 ± 0.5 A	0.2 ± 0.1 A
	PG x 'Bluebird'	H2012-030-01	5.9	6	5	83	28	5.6 ± 0.8 A	4.7 ± 1.1 A
	PG x Fiji™	H2013-129-08	5.6	30	21	70	76	3.6 ± 0.4 A	2.5 ± 0.4 A
	PG x Lil' Kim™	H2013-084-21	6.0	31	4	13	3	0.8 ± 0.3 A	0.1 ± 0.1 A
	PG x 'Red Heart'	H2013-085-01	6.1	29	6	21	6	1.0 ± 0.4 A	0.2 ± 0.1 A
Control – 4*x* × 4*x* ♀ fertility estimates								18 ± 2 B	9.9 ± 1.4 B
	Blue Satin®	--	--	107	75	70	1626	22 ± 1	14.1 ± 2.6
	'Blue Bird'	--	--	240	109	45	1798	17 ± 1	8.3 ± 2.2
	'Buddha Belly'	--	--	42	34	81	752	22 ± 1	19.2 ± 3.1
	'Diana'	--	--	395	87	22	1302	15 ± 1	5.6 ± 1.5
	Lil' Kim™	--	--	74	28	38	440	16 ± 1	5.3 ± 2.4
	'Minerva'	--	--	40	22	55	311	14 ± 2	6.3 ± 2.5
	'Red Heart'	--	--	173	62	36	1720	28 ± 2	9.8 ± 2.9
	'Woodbridge'	--	--	115	45	39	805	18 ± 1	8.5 ± 2.5

Table 7.7 (continued). Pollination, capsule, and seed estimates from testcrosses on progeny resulting from combinations of tetraploid and hexaploid *Hibiscus syriacus* L.

Treatment[z]	♀ pedigree[y]	♀ 5*x* accession no.	♀ 2C genome size (pg)[x]	Pollinations (no.)	Capsules (no.)	Pollination (%)[w]	Seeds (no.)	Seeds·capsule^{-1} (mean ± SE)[v]	Seeds.pollination-1 (mean ± SE)u
	Bali™	--	--	110	78	71	1443	19 ± 1	11.5 ± 2.6
	Blue Chiffon™	--	--	127	76	60	1410	19 ± 1	11.5 ± 2.6
	Fiji™	--	--	47	25	53	383	15 ± 1	9.3 ± 1.9
	Tahiti™	--	--	39	6	15	29	5 ± 1	1.1 ± 0.5
	White Chiffon®	--	--	40	25	63	690	28 ± 1	18.6 ± 4.8

[z]Tetraploid (4*x*) and hexaploid (6*x*) cross combinations. Controls are represented by single and semi-double female fertility estimates from 4*x* × 4*x* crosses with 5+ male pollinizers and 30+ pollinations.

[y]Pedigree of interploid hybrids between hexaploid Pink Giant (PG) and tetraploid taxa. Controls include open-pollinated (OP) seed from three fertile tetraploid taxa.

[x]Estimate of holoploid 2C genome size.

[w]Percent pollination calculated per accession as: [total filled capsules / total pollinations]*100.

[v]Seedlings per pollination calculated for each treatment (within boxes) and per accession. For each accession, seedlings per pollination was calculated as: [total seedling / total pollinations] × 100. For each treatment, average seedlings per pollination were calculated from accession estimates with at least three pollinations. Letters separating least squares (LS) means based on comparison lines test of the generalized mixed model procedure (GLIMMIX).

[u]Percent germination calculated for each treatment (within boxes) and per accession. For each accession, percent germination was calculated as: [total seedlings / total seeds] × 100. For each treatment, average percent germination was calculated from accession estimates with 10+ seed.

Table 7.8. Pollination, germination, and seedling estimates from testcrosses on progeny resulting from combinations of tetraploid and hexaploid *Hibiscus syriacus* L.

Treatment[z]	♀+ pedigree[y]	♂+ 5*x* accession no.	♂+ 2C genome size (pg)[x]	Pollinations (no.)	Capsules (no.)	Pollination (%)[w]	Seeds (no.)	Seedlings (no.)	Seedlings·pollination^{-1} (mean ± SE)[v]	Germination (%)[u]
4*x* x 6*x*									0.8 ± 0.3 A	45 ± 4 A
	‘Aphrodite’ x PG	H2012-005-01	6.4	51	34	67	188	71	1.4	38
		H2013-017-21	5.7	12	9	75	27	14	1.2	52
		H2013-017-16	6.1	58	23	40	40	22	0.4	55
	‘Bluebird’ x PG	H2012-011-02	5.8	2	0	0	0	0	MV	MV
		H2012-011-04	6.3	3	2	67	0	0	0.0	MV
		H2012-011-07	6.1	24	7	29	4	0	0.0	0
	‘Diana’ x PG	H2013-049-01	5.7	3	1	33	0	0	0.0	MV
	‘Helene’ x PG	H2013-124-13	6.1	81	77	95	552	253	3.1	46
		H2013-124-19	5.9	14	9	64	5	1	0.1	20
		H2013-124-03	6.3	29	27	93	64	22	0.8	34
	‘Woodbridge’ x PG	H2012-041-01	6.8	1	1	100	1	0	MV	0
6*x* x 4*x*									1.4 ± 0.9 A	45 ± 14 A
	PG x ‘Aphrodite’	H2013-077-05	5.6	38	23	61	289	206	5.4	71
	PG x Bali™	H2013-078-03	5.7	21	4	19	5	2	0.1	40
	PG x ‘Bluebird’	H2012-030-01	5.9	6	5	83	28	12	2.0	43
	PG x Fiji™	H2013-129-08	5.6	30	21	70	76	17	0.6	22
	PG x Lil’ Kim™	H2013-084-21	6.0	31	4	13	3	2	0.1	67
	PG x ‘Red Heart’	H2013-085-01	6.1	29	6	21	6	3	0.1	50
Control – OP Seeds										89 ± 1 B
	Blue Satin®	11-0210	4.7	--	--	--	50	43	--	86
	White Chiffon®	13-0044	4.7	--	--	--	50	45	--	90
	‘Woodbridge’	11-0214	4.7	--	--	--	50	45	--	90

[z]Tetraploid (4*x*) and hexaploid (6*x*) cross combinations. Controls represent open-pollinated (OP) seed from tetraploid cultivars.

[y]Pedigree of interploid hybrids between hexaploid Pink Giant (PG) and tetraploid taxa. Controls include open-pollinated (OP) seed from three fertile tetraploid taxa.

[x]Estimate of holoploid 2C genome size.

[w]Percent pollination calculated per accession as: [total filled capsules / total pollinations]*100.

[v]Seedlings per pollination calculated for each treatment (within boxes) and per accession. For each accession, seedlings per pollination was calculated as: [total seedling / total pollinations] × 100. For each treatment, average seedlings per pollination were calculated from accession estimates with at least three pollinations. Letters separating least squares (LS) means based on comparison lines test of the generalized mixed model procedure (GLIMMIX).

[u]Percent germination calculated for each treatment (within boxes) and per accession. For each accession, percent germination was calculated as: [total seedlings / total seeds] × 100. For each treatment, average percent germination was calculated from accession estimates with 10+ seed.

Figures

Fig. 7.1. Pollination and novel floral phenotypes of pentaploid accessions of Hibiscus syriacus. (A) Daily pollinations of pentaploid accessions using flowers collected from randomly from proven male fertile cultivars. (B) Novel, pink, rose-like, bicolor accession (H2013-129-08) resulting from the cross 'Pink Giant' x Fiji™. (C) Novel, fully-double accession (H2013-131-06) with petaloid female and male whorls from the cross 'Pink Giant' x 'Blushing Bride'.

Fig. 7.2. Bi-color Fiji™ floral phenotype in *Hibiscus syriacus* expression in pentaploid and tetraploid seedlings. (A) Pentaploid hybrid (H2013-129-08) resulting from the cross 'Pink Giant' x Fiji™ exhibiting bi-color petals on semi-closed flowers. (B) Tetraploid hybrid (H2013-059-09) from the cross Fiji™ x White Chiffon® exhibiting the standard, bi-color Fiji™ phenotype on fully opened flowers.

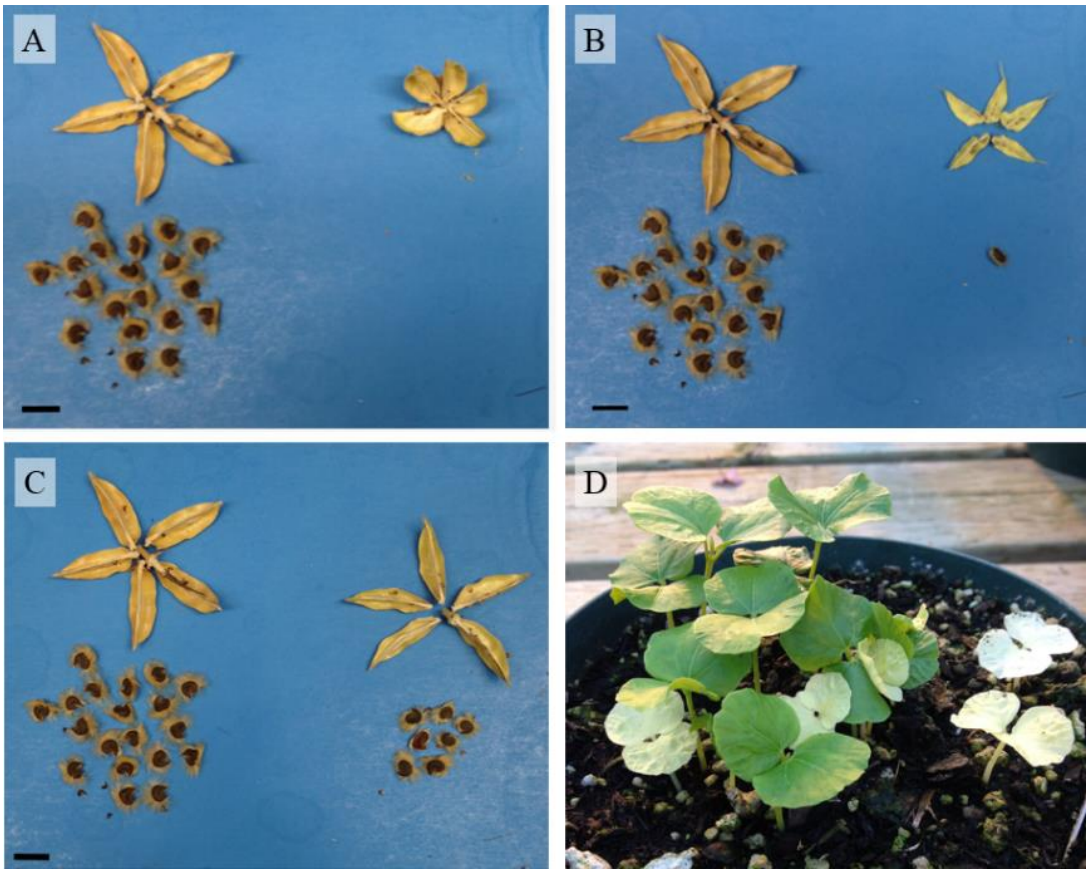

Fig. 7.3. Seed and seedling development from testcrosses of pentaploid *Hibiscus syriacus*. Scale bar = 1 cm. (A) Fruit from open-pollinated *H. syriacus* 'Woodbridge' (left) and pentaploid hybrid *H. syriacus* 'Blue Bird' × 'Pink Giant' (right) pollinated with *H. syriacus* 'Red Heart'. (B) Fruit from open-pollinated *H. syriacus* 'Woodbridge' (left) and pentaploid hybrid *H. syriacus* 'Helene' × 'Pink Giant' (right) pollinated with *H. syriacus* Lil' Kim™. (C) Fruit from open-pollinated *H. syriacus* 'Woodbridge' (left) and pentaploid hybrid *H. syriacus* 'Helene' × 'Pink Giant' (right) pollinated with *H. syriacus* 'Woodbridge'. (D) Germinating fertility testcross seeds from pentaploid hybrid *H. syriacus* 'Pink Giant' × 'Aphrodite' pollinated with *H. syriacus* 'Diana' exhibiting albino seedlings.

CHAPTER 8: SEGREGATION OF FLORAL TRAITS IN ALTHEA (*Hibiscus syriacus* L.)

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Additional index words: CIEL*a*b*, eyespot, recessive, dominant, homozygous, heterozygous, quantitative, anthocyanin, petaloid stamen, petal area

Abstract. Althea (*Hibiscus syriacus*) has been a staple ornamental shrub prized for its winter hardiness, range of flower colors, and unique flower forms, including single-flowered types and double-flowered types with petaloid stamens. Although floral traits are most important for breeders of althea, little is known about their segregation patterns. The objectives of this study were to determine segregation patterns in eyespot presence, flower color, and flower form among progeny of elite cultivars. Over four years, thousands of pollinations were attempted, yielding just over 3100 flowering seedlings for observation of F_1, F_2, and backcross families. For each plant, data on one or two flowers was collected including presence vs. absence of eyespot, estimates of flower size including petal area (length x width), petal number (five true petals + petaloid stamen), and petal body color (CIEL*a*b*). A utility was also created to convert color measurments into color values on the Royal Horticulture Society Colour Chart. Based on previous research in other species of *Hibiscus*, pure white flowers without eyespots was hypothesized to be a recessive

trait. Recessive testcrosses and chi-square analyses were performed among three taxa ('Buddha Belly', 'Diana', and White Chiffon®), and between this recessive group and a suite of colorful cultivars. Self-pollinations and intercrosses within putative homozygous dominant and putative homozygous recessive groups further confirmed their genotypes. Based on these results, we propose that eyespot is controlled by a single gene called *spotless*, named for the recessive allele which results in a complete elimination of color in flowers. Crosses that resulted in progeny that all produced eyespots were observed to segregate for color in the petal body, with one group (white to blush pink) appearing to be recessive to full color. Recessive testcrosses and chi-square analyses were performed among nine taxa exhibiting eyespots with white to blush petal bodies, and between taxa with fully colorful petal bodies. These testcrosses resulted in a putative homozygous dominant group composed mostly of blue and dark pink taxa, while the heterozygous group was composed mostly of pink taxa. *Spotless* taxa were also added to these two groups, suggesting an epistatic interaction with the *spotless* allele. Based on these results, we propose that petal body color is controlled by a single gene called *geisha*, named for the recessive allele which produces white to blush-pink petal bodies and dark red eyespot. This trait exhibits incomplete dominance and is under epistatic control by *spotless*. *Geisha* type flowers lack pigment in the petal body, or exhibit a blush, likely produced by low levels of cyanidin, peonidin, and pelargonidin. The interaction and segregation of these two genes was additionally confirmed in F_1, F_2, and backcross families from two crosses: Lil' Kim™ x Blue Chiffon™ and Fiji™ x White Chiffon®. In addition to color segregation, depth of color irrespective of hue (CIE L*) was also investigated

in seedlings of a wide range of crosses (*spotless* and *geisha* types not included). Individual crosses had a significant effect on average color depth (CIE L*) ($P < 0.0001$). The deepest pigments were measured in crosses among hexaploid 'Pink Giant', taxa homozygous dominant for *geisha*, and taxa heterozygous for *geisha*. Conversely, the lightest pigments were observed in crosses between *geisha* taxa and taxa heterozygous for *geisha*. Therefore, future efforts at eliminating the *geisha* allele from a breeding population may allow for quantitative improvement in total anthocyanin production. Interploid hybridization with advanced selections may further increase pigment production with the added benefit of reducing fertility. Observations revealed that progeny produced a continuous distribution of petal number between the petal numbers of the two parents, with occasional transgressive segregants in each population. Petal numbers varied significantly by cross ($P < 0.0001$) and cross type ($P < 0.0001$), including single single- x single-flowered reciprocal crosses (S | S), single- x double-flowered reciprocal crosses (S | D), and double- x double-flowered reciprocal crosses. The highest average petal numbers across all cross types were found in (D | D) crosses at 36.0 ± 2.4 petals, followed by (S | D) crosses at 13.9 ± 1.0 petals, and (S | S) crosses with 5.9 ± 0.2 petals Therefore, quantitative improvement on petal number may be possible by selecting and recombining progeny with heavy petal production over successive generations. Among all traits, petal size was the only trait to vary widely by environment and by year. Flower size, as measured by petal area (length x width), varied significantly among crosses ($P < 0.0001$) and cross type (S | S), (S | D), and (D | D) in *H. syriacus* ($P = 0.0020$). Flower size of progeny resulting from (S | D) cross types was found to

be significantly larger than both (S | S) and (D | D). However, further work must be undertake to eliminate environmental effects from flower size estimates. This study represents a comprehensive investigation into the segregation of floral traits in *H. syriacus*, and will benefit future efforts to improve flowers in this vibrant ornamental shrub.

Introduction

Hibiscus L. is a genus belonging to Malvaceae, which represents approximately 250 species of trees, shrubs and herbs (Van Laere, 2008). Rose-of-sharon or althea (*H. syriacus* L.) has been a staple ornamental shrub in American gardens prized for its winter hardiness, range of flower colors, and unique flower phenotypes including single-flowered and double-flowered forms (Contreras and Lattier, 2014). Double-flowered taxa can produce a wide range of petaloid stamen, and have been previously categorized as anemone (semi-double) or fully double types (Contreras and Lattier, 2014). Breeders have noted the potential for improvement in *H. syriacus* due to their range of flower color and form and their short generation time from seed to flower (Dirr, 2009). However, no formal study has determined the inheritance patterns of floral traits.

The basic chromosome number of *H. syriacus* has been reported as $x = 20$ with most cultivars being tetraploid, $2n = 4x = 80$ (Skovsted, 1941). Polyploidy has been investigated in *Hibiscus* section *Furcaria* with tetraploids, hexaploids, octaploids, and decaploids all exhibiting allopolyploidy (Menzel and Wilson, 1969; Wilson, 1994, 1999). Tetraploids of section *Furcaria* have been discovered to be

allopolyploids including *H. acetosella* (AABB, 2*n* = 4*x* = 72) and *H. radiatus* (AABB, 2*n* = 4*x* = 72) (Satya et al., 2012). Chromosomes of allopolyploids (amphidiploids) usually pair as bivalents allowing simply inherited traits (perhaps flower color) to segregate as diploids, simplifying interpretation of inheritance patterns. Adding further evidence to disomic inheritance in *Hibiscus*, oryzalin-induced autoallooctaploid *Hibiscus acetosella* proved to be completely sterile as both a pistillate and staminate parent in controlled crosses (Contreras et al., 2009). Inducing autopolyploidy in allopolyploid *Hibiscus* may increase the number of multivalents, which decreases fertility (Contreras et al., 2009). Though it remains unclear if *H. syriacus* (section *Hibiscus*) shares ancestral allopolyploid with its relatives in section *Furcaria*, understanding inheritance patterns of floral traits could aid breeders in creating novel cultivars of *H. syriacus*.

Interpreting segregation patterns in tetraploid progeny of *Hibiscus* could prove difficult. Not only is disomic or polysomic inheritance possible, but the number of genes controlling the phenotype of interest also must be taken into consideration. Scant research has been conducted on genetic control of traits in *Hibiscus*, such as the double-flowering (petalloid stamens). Inheritance of the double flowering trait has been investigated in many taxa and can be dominant or recessive, with dominance being incomplete in taxa such as *Gerbera*, *Pelargonium*, and *Petunia*, and with dosage effects occurring in polyploids according to the number of alleles present (Vainstein, 2002). More than one gene has been shown to control double flowering with three genes controlling doubling in *Pelargonium* (Almouslem and Tinley-Basset, 1989) and two genes controlling flowering in *Cosmos* (Samata, 1958) and *Begonia*

(Vainstein, 2002). Although double flowering has proven to be a lucrative trait for ornamental *Hibiscus*, no studies have investigated inheritance of double flowers.

In addition to flower form, flower color is a top priority for breeders of *H. syriacus*. Genetic control of flower color in ornamental plants has been investigated in prior research, and control of flower color can often involve more than one locus. Yue et al. (2008) found that flower color in *Helianthus* was controlled by two independent loci while Griesbach (1996) found that flower color in *Petunia* was controlled by four loci. Getty (2012) investigated the number of loci, number of alleles, and gene action controlling flower color in the diploid *H. coccineus* Walter. After conducting numerous crosses and evaluating F_1, F_2, and backcross progeny, data revealed that flower color was controlled by two alleles at one locus with white flower color being recessive to red (Getty, 2012). If flower color in *H. syriacus* is also controlled by one locus, with white being recessive, then segregation patterns should be discernable in either polysomic or disomic inheritance in *H. syriacus*.

Petals of *Hibiscus syriacus* are characterized by ivory or anthocyanin pigments (Fig. 8.1) in the main petal body with a red eyespot (sometimes "eye spot") at the base (Kim et al., 1989a). Specific anthocyanins are produced by several different routes in the flavonol biosynthetic pathways (Petrussa et al., 2013). Different levels and compositions of anthocyanins in *H. syriacus* are responsible for color variation in the petal body, including red and pink pigments from cyanidin and peonidin; pink pigments from pelargonidin; and dark pink, lavender, and blue pigments from delphinidin, petunidin, and malvidin (Kim et al., 1989a). Anthocyanin

levels are elevated in the eye spot compared with the main body of the petal, with cyanidin derivatives making up the main eye spot pigments (Kim et al., 1989a).

The purpose of eyespots, whether pigmented or ultraviolet, are usually to signal the presence of a pollination reward near the base of the flower (Koski and Ashman, 2013). Variation in eyespot size in *Hibiscus* has been previously used to select new cultivars, including the enlarged eyespot in *H. syriacus* 'Red Heart'. More recently, interspecific hybridization has been used to develop novel hybrids with long eyespots (Ha et al., 2010, 2015). Studies on inheritance of ultraviolet patterns in *Argentina anserina* (Koski and Ashman, 2013) and *Brassica rapa* (Syafaruddin, 2006) suggests that eyespot size is a floral trait that may respond to selection.

In addition, induced mutations in *Mimulus lewisii* that created flowers lacking nectar guides (*guideless*) were observed to segregate as a Mendelian recessive trait (Owen and Bradshaw, 2011; Yuan et al., 2013). Segregation of eyespot presence vs. absence in *H. syriacus* has yet to be investigated, but may involve a mutation in the flavanol pathway that completely eliminates pigment production. No cultivars currently exist with pigmented petals that also lack an eyespot, indicating that lack of an eyespot may be a mutation upstream in the pigment biosynthesis pathway. Several popular taxa currently sold in the nursery trade lack an eyespot including the single flowering *H. syriacus* 'Diana' and the semi-double flowering *H. syriacus* White Chiffon®. Another notable taxa lacking an eyespot is *H. syriacus* 'Buddha Belly', a rare selection with swollen, caudiform growth of the lower trunk, swollen nodes, and stiff upright stems.

Flower size, petal number, and presence vs. absence of eyespot are relatively straightforward floral characteristics that require simple observations and measurements. However, color is often an enigmatic trait that defies simple measurement. Currently, the most accurate method of color measurement is the CIEL*a*b* color space, adopted by the International Commission on Illumination (Commission internationale de l'éclairage) in 1976. It has been applied to diverse types of research such as variation in wine pigmentation among grape varieties (García-Marino et al., 2012, 2013), crop segmentation in rice fields (Bai et al., 2013), and variation in resistance to fungal infection in tropical forest trees (Okino et al., 2015). Quantitative measurements of flower colors have been performed on ornamental plants including dried flowers, such as in Dutch roses (Bintory, 2015), and fresh flowers, such as carnations (Gonnet, 1993). However, no known study exists showing quantitative color measurements in *H. syriacus*.

In addition, the most common color scale used for flower color is the RHS Colour Chart, which is not quantitative and not directly comparable to CIEL*a*b* values. Flower colors of *H. syriacus* fit into broad categories of white, pink, lavender, and blue, but segregating populations in a breeding program rarely fit easily in such color categories. Any effort to categorize color using the RHS Colour Chart will be impaired by subjectivity of the observer and will require the same environment and lighting for accurate measurements (Gonnet, 1993; 1995). However, efforts to obtain colorimetric measurements of the 800+ color chips in the RHS Colour Chart have shown promise for being able to easily convert between the

qualitative scale of the RHS Colour Chart and the quantitative scale of CIEL*a*b* (Gonnet, 1995).

The objectives of this study are to determine segregation patterns in eyespot, flower color, and flower form among progeny resulting from crosses of elite cultivars of *H. syriacus*.

Methods and Materials

Plant materials. To test segregation of floral traits in *H. syriacus*, elite cultivars were collected from botanical gardens, arboreta, and nurseries (Table 8.1). Both potted plants as well as cuttings were acquired. Plants were grown at Oregon State University and mature plants were grown at the Lewis Brown Horticulture Farm (Corvallis, OR). For each taxon, original cultivar and trademark names were maintained from each source. However, for *H. syriacus* and many ornamental taxa, usually one name becomes common in the nursery trade as the 'market name'. For simplicity, only market names (cultivar or trademark) will be used hereafter.

Crosses. Crosses were made in summer in a glasshouse kept free of pollinators with day/night temperatures of 25/20 °C and a 16-h photoperiod (Fig. 8.2A). Flowers were open for two days before stigmas reflexed in an effort to self-pollinate. Therefore, flowers were pollinated in the morning of their first flowering and stigmas were thoroughly covered with a dense layer of pollen. Fresh pollen was collected on the day of pollination. Pollen of *H. syriacus* is large (108 to 169 μm) which prevents it from becoming airborne (Bae et al., 2015). Pollen grains also produce numerous, long, sticky spines from their exine. These 28 to 84 spines per

grain with spine lengths of 8 to 25 μm cause the pollen to clump (Bae et al., 2015). Therefore, for pollination, clumps of pollen were placed on stigmas with forceps. Forceps were sterilized in 70% ethanol between pollinations. When flowers were abundant, pollinations were performed directly using the monadelphous stamen of the male parent. Each cross was labelled with a jeweler's tag on which was recorded the parents and date (Fig. 8.2B). Each cross was observed daily for capsule development or flower abortion (Fig. 8.2B).

For successful crosses, capsules were cleaned and non-stratified seeds from each cross were sown into 1.3-L containers filled with growing medium (Metro-Mix; Sun Gro Horticulture, Agawam, MA) in seed lots of $\leq$ 30 seeds per container. Surviving seedlings were transplanted into 2.5-L containers filled with douglas-fir-based potting substrate during summer and grown under conditions described above. Due to space limitations, plants were grown and evaluated in several glasshouses, two polyhouses, and two field locations. Due to variation in growth rates and flowering, plants were evaluated over multiple years (2013-2016). Floral traits were assumed to be highly heritable and no blocking was done to determine variation in response to environmental effects or years.

Eyespot. Due to previous reports of disomic inheritance in *Hibiscus* and reports that lack of an eyespot is often inherited as a simple Mendelian recessive trait, self-pollinations and reciprocal testcrosses were conducted using three taxa that lack an eyespot, 'Buddha Belly', 'Diana', and White Chiffon®. Crosses were conducted on dozens of taxa producing eyespots, and seedlings were grown and evaluated over multiple years according to the methods listed above. Self-pollinations of 'Buddha

Belly', 'Diana', and White Chiffon® were expected to have only flowers that lack eyespots. Crosses of *spotless* cultivars with cultivars that contain eyespots were expected to have a 1:0 ratio of eyespot : *spotless* for homozygous dominant parents or 1:1 ratios for heterozygous parents. For cultivar groups designated as putative heterozygotes for the eyespot trait, self-pollinations and cross-pollinations were performed with an expected 3:1 segregation of eyespot : *spotless*.

Flower color. Color measurements were collected for each cultivar and hybrid using a portable colorimeter (BC-10; Konica Minolta, Tokyo, Japan) that reports in the CIE L*a*b* scale. The CIE L*a*b* scale is a Cartesian coordinate system that represents lightness (L*) with values from 0 (black) to 100 (white), red/green color opposition (a*) with values from -100 (green) to 100 (red), and yellow/blue color opposition (b*) with values from -100 (blue) to 100 (yellow) (García-Marino, 2012). One to two petals were randomly collected from each plant and measured during peak bloom in late summer (Fig. 8.3A). Petal color was only measured on fresh flowers on their first day (of the two day flowering period for *H. syriacus*) due to changes in petal color during senescence (Kim et al., 1989b). Measurements were taken in the center of the petal body between the eyespot and distal end of the petal. Prior to measurement, the colorimeter was calibrated using a pure white calibration plate (Konica Minolta) and petals were measured on the calibration plate to standardize the background and remove any variation from petal translucence. Individual values for L*, a*, and b* were recorded and averaged together to estimate the color for each individual taxon.

Because CIE L*a*b* is not the most widely used color scale for flower colors, CIE L*a*b* values were converted to the nearest RHS Colour Chart (Royal Horticultural Society, 2007) value by creating a macro utility (Excel; Microsoft, Redmond, WA) (Fig. 8.4). To create this utility, the entire 884 colors in the RHS Colour Chart were measured with a colorimeter (Konica Minolta). Each color was measured three times in three different locations across the color chip and an average was calculated for each color. The macro utility works by calculating color differences between a sample and each reference value of the RHS Colour Chart according to the following color difference equation:

$$\Delta E^* = \sqrt{[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]}$$

where samples are compared to each reference (R),

$$\Delta L^* = L^*_1 - L^*_R$$

$$\Delta a^* = a^*_1 - a^*_R$$

$$\Delta b^* = b^*_1 - b^*_R$$

Then, the macro utility reports back the nearest RHS value and a colored cell (Excel, Microsoft) representing the color (Fig. 8.4). Since custom colors generated in Excel follow the RBG color system, each CIE L*a*b* value of the RHS Colour chart was converted from CIE L*a*b* to RBG using an open-source color converter (colormine.org) to create each custom colored cell. Due to copyright restrictions, the macro utility created for this project cannot be released as open-source.

Flower size. Early observations of hybrid flowers showed variation in the angle of attachment of each flower petal. In addition, the angle of attachment varied

throughout the two-day bloom time. Therefore, a simple measurement of total flower width proved elusive. Petal length provided a more accurate measure of flower size, yet early observations in hybrids revealed large variation in petal width. Some hybrids produced wide petals that overlapped heavily, adding to the visual display. Previous breeding programs have selected for novel traits such as wide petals and ruffled petals, yielding new cultivars of *Hibiscus* (Ha et al., 2010, 2014, 2015). Therefore, a petal area was calculated for each taxon as an estimate of flower size (Fig. 8.3B). Each petal was laid on a flat surface and the length of the petal was measured from the base to the furthest point. Petal width was measured across the widest two points. One to two petals were randomly collected from each plant and measured during peak bloom in late summer. These taxon estimates were averaged together to obtain an average petal size for each cross.

Petal number. Early observations of hybrids revealed wide variation in petal number in crosses with double-flowering taxa. Even single flower crosses could occasionally produce a few, small petaloid stamens. Therefore, hybrids were not easily classifiable into simple flower form groups such as single, semi-double, and double. Therefore, to obtain a quantitative measurement for each hybrid, average petal numbers were counted for each taxon. One to two flowers were randomly collected from each plant and measured during peak bloom in late summer. All petals for each flower were removed and counted (Fig. 8.3C). These taxon estimates were averaged together to obtain an average petal numbers for each cross.

Statistical analyses. Segregation of flower color traits were investigated by χ^2 goodness of fit tests to compare expected ratios for F_1, F_2, and backcross progeny to

observed ratios. For single-gene traits that segregated in simple amphidiploid ratios (1:0, 0:1, 3:1, etc.), diploid (rather than tetraploid) genotypes are reported for simplicity. Due to cross- and self-incompatibility of among cultivars (Chapter 7), and disease outbreaks and winter dieback in numerous progeny, the original cross design of a full diallel did not yield enough data to estimate genetic variability within the breeding population for quantitative traits such as petal size and petal number. Therefore, average petal number and petal size for each cross were reported and subjected to a mixed model analysis of variance (ANOVA) (SAS Studio; Cary, NC). In addition, cross means for petal number and petal size were used to generate means for flower form combinations (single x single, single x double, and double x double). Means were separated for individual crosses as well as flower form combinations using the LINES command of the generalized linear mixed model procedure (GLIMMIX) (SAS Studio; Cary, NC).

Results and Discussion

Eyespot. Cuticle peels and flower dissections revealed that pigment production in flowers of *H. syriacus* was isolated to the cuticle layer in the petal body and eyespot (Fig. 8.5). Entire cuticle cells expressed pigment, likely due to vacuolar pigment accumulation, with a marked increase in pigment production at the transition between the petal body and eyespot (Fig. 8.5). Only three parent taxa used in crosses lacked an eyespot, ‘Buddha Belly’, ‘Diana’, and White Chiffon®. A total of 246 seedlings were evaluated from self-pollinations and intercrosses among these cultivars. Not a single progeny produced any pigment, including an eyespot, yielding

a 0:1 (eyespot : *spotless*) segregation ratio (Table 8.2). The only self-compatible taxon in the *spotless* group was White Chiffon®. All 74 S_1 seedlings of White Chiffon® were *spotless*. Based on these results, the three *spotless* taxa were used as testers in recessive testcrosses with a wide range of cultivars exhibiting eyespots (Fig. 8.6). Reciprocal combinations with testers that yielded seedlings segregating 1:0 (eyespot : *spotless*) were grouped together as putative homozygous dominant for eyespot. This group included 'Aphrodite', Bali™, Fiji™, Lavender Chiffon™, Lil' Kim™, 'Lucy', Pink Chiffon®, and Strawberry Smoothie™ (Fig. 8.6). Reciprocal combinations with testers that yielded seedlings with a segregation ratio of 1:1 (eyespot : *spotless*) were grouped together as putative heterozygotes for eyespot. This group included all blue-flowering taxa ('Blue Bird', Blue Chiffon™, Blue Satin®) as well as 'Minerva', 'Red Heart', and 'Woodbridge' (Fig. 8.6). The reciprocal cross between Blue Satin® and 'Buddha Belly' produced the most progeny (168) for observation, resulting in a segregation of 1:1 (eyespot : *spotless*) ($\chi^2 = 0.214$, $P = 0.643$) (Table 8.2). Only one cross between Blue Satin® and 'Diana' yielded segregation ratios that diverged from the expected 1:1 ratio, possibly a result of accidental self-pollination or mislabeling (Table 8.2).

Within the putative homozygous dominant group, self-pollinations and intercrosses were used to further confirm these taxa were not heterozygous. The total 276 progeny resulting from self-pollination and intercrosses among putative homozygous dominant taxa resulted in 1:0 (eyespot : *spotless*) segregation ratios (Table 8.2). In an additional step to confirm the putative homozygous dominant group, intercrosses were performed between these taxa and putative heterozygotes

yielding segregation ratios of 1:0 (eyespot : *spotless*) in the 478 progeny evaluated. Only ten *spotless* seedlings were recorded in these crosses, with six observed in combinations between putative homozygous dominant 'Aphrodite' and putative heterozygotes 'Woodbridge', Blue Satin®, and 'Blue Bird' (Table 8.2). Four *spotless* seedlings were recorded from the cross 'Lucy' x 'Red Heart' (Table 8.2). These few recorded *spotless* seedlings could be the result of accidental self-pollinations, chance mutations, or the result of mislabeling. Two additional taxa with no or low numbers of progeny from the recessive testcrosses were added to the putative homozygous dominant group based on crosses with putative heterozygotes (Fig. 8.6). China Chiffon™ and 'Blushing Bride' were categorized as putative homozygous dominant after an evaluation of a combined 114 progeny resulting from testcrosses with putative heterozygotes and yielding segregation ratios of 1:0 (eyespot : *spotless*) (Table 8.2).

Within the putative heterozygous group, self-pollinations and intercrosses were used to further confirm these taxa were heterozygous. All self-pollinations yielded segregation ratios of 3:1 (eyespot : *spotless*) with 'Woodbridge' proving the most self-fertile with 66 seedlings observed ($\chi^2 = 0.314$, $P = 0.575$) (Table 8.2). Of the intercrosses, reciprocal combinations between Blue Chiffon™ and 'Red Heart' yielded the most progeny for observation (108) and a segregation ratio of 3:1 (eyespot : *spotless*) ($\chi^2 = 0.790$, $P = 0.374$) (Table 8.2). Only one cultivar, Hawaii™, was not initially included in the recessive testcrosses but was categorized as a putative heterozygote based on self-pollinations (Fig. 8.6). Self-pollinations of Hawaii™ revealed a segregation ratio of 3:1 (eyespot : *spotless*) ($\chi^2 = 0.067$, $P =$

0.796) (Table 8.2). However, only five plants flowered and further confirmation of Hawaii™ as a heterozygote will be necessary.

Based on these combined results, we propose that the presence or absence of an eyespot is controlled by a single recessive allele (named *spotless*) with eyespot exhibiting complete dominance in the heterozygous taxa. No flowers across the entire progeny population were observed to have a colorful petal body combined with a white (absent) eyespot. This indicates that the *spotless* gene is likely upstream in the flavonoid biosynthetic pathway (Fig. 8.1) resulting in a complete elimination of all flower color. Although *H. syriacus* is reported to be a tetraploid, the *spotless* phenotype segregates according to simple Mendelian diploid inheritance patterns. Therefore, for simplicity, we propose the diploid genotype notation of *ss* to represent the *spotless* phenotype and (*SS*, *Ss*) to represent the genotypes for eyespot. All blue-flowering taxa, including Blue Satin®, 'Blue Bird', and Blue Chiffon™, were found to be heterozygotes and carry the *spotless* allele. In contrast, the majority of taxa categorized as homozygous dominant exhibit white to blush pink petal bodies (Fig. 8.6). Considering the flavonol pathway from Petrussa et al. (2013), perhaps having only one copy of the eyespot gene in the cyanidin pathway allows more precursors for the pelargonidin, delphinidin, petunidin, and malvidin pathways, resulting in deeper blue or pink/lavender flowers, as observed in the blue taxa and the other heterozygote, 'Woodbridge'. However, one white flower was discovered that also carried the *spotless* allele, 'Red Heart', while colorful taxa such as 'Aphrodite', Lavender Chiffon™, and 'Lucy' proved to not carry *spotless* (Fig. 8.6). Clearly other

genes and gene interactions are involved in determining the specific hue of the petal body.

Flower color. To begin determining segregation of color in the petal body, crosses that failed to yield *spotless* progeny (*SS* x *SS*, *SS* x *Ss*, and *SS* x *ss*) were first observed for additional segregation patterns in petal color. By sorting the progeny based on a quantitative estimate of color depth (CIE L*), segregation patterns began to emerge between plants that produced full color pigment in the petal body ("colorful" hereafter) and plants that produced little to no color in the petal body (Fig. 8.7). Elite cultivars that exhibited flowers with a bright red eyespot and white, bicolor, or blush pink petal bodies ("*geisha*" hereafter) were observed to produce only progeny with *geisha* phenotypes in intercrosses and self-pollinations. Therefore, this group was used as testers to explore the possibility of a recessive allele that reduces pigment production in the petal body (Fig. 8.8). For segregation tests on the *geisha* phenotype, all progeny exhibiting the *spotless* phenotype were removed from the analyses. Only six combinations yielded unclear segregation patterns, likely due to low numbers of seedlings, accidental self-pollinations, and mislabeling (Table 8.3).

A total of 253 seedlings were evaluated from self-pollinations and intercrosses among the putative homozygous recessive cultivars for the *geisha* phenotype (Table 8.3). Not a single seedling resulting from the self-pollinations and intercrosses in this group produced fully colorful petals, resulting in a segregation ratio of 0:1 (colorful : *geisha*) (Table 8.3). Initially, all white-flowered *spotless* taxa were included in the putative recessive group, but White Chiffon® and 'Buddha Belly' were found to produce colorful progeny when crossed with other taxa in the putative recessive

group (Table 8.3). This result is likely due to recessive epistasis of the *spotless* gene over the genes control petal body color. The only *spotless* taxon that produced segregation ratios of 0:1 (colorful : *geisha*) in recessive testcrosses was 'Diana'. Therefore, the final putative recessive testcross group was determined to include Bali™, 'Blushing Bride', 'Diana', Fiji™, 'Helene', Lil' Kim™, Pink Chiffon®, and 'Red Heart' (Fig. 8.8). An additional taxon, Strawberry Smoothie™, was later added to the recessive group based on testcrosses with the putative heterozygous group (Fig. 8.8). Another likely member of the recessive group, China Chiffon™, was not included due to a lack of appropriate crosses and low numbers of progeny (Table 8.3). Further efforts will be necessary to confirm China Chiffon™ as homozygous recessive.

These taxa were used in as testers in recessive testrcrosses with a wide range of cultivars exhibiting colorful and *spotless* phenotypes (Fig. 8.8). Reciprocal combinations with testers that yielded seedlings exhibiting a 1:0 segregation (colorful : *geisha*) were classified as putative homozygous dominant (Fig. 8.8). These taxa included all blue-flowered taxa ('Blue Bird', Blue Chiffon™, Blue Satin®, and Hawaii™), one *spotless* taxon ('Buddha Belly'), and one pink-flowered taxon ('Lucy') (Fig. 8.8). Reciprocal combinations with testers that yielded seedlings exhibiting a 1:1 segregation ratio (colorful : *geisha*) were classified as putative heterozygotes (Fig. 8.8). These taxa included pink-flowered taxa ('Aphrodite' and 'Woodbridge'), pink-lavender taxa ('Minerva' and Lavender Chiffon®), and one *spotless* taxon (White Chiffon®).

Recessive testcrosses between the *geisha* group and the putative heterozygous group yielded a segregation ratios of 1:1 (colorful : *geisha*) for all cross combinations (Fig. 8.8). Out of a total of 397 total progeny evaluated, the most prolific crosses with 1:1 segregation (colorful : *geisha*) was between 'Aphrodite' and 'Diana', yielding 81 seedlings (χ^2 = 0.111, *P* = 0.739) and the cross Pink Chiffon® x White Chiffon®, yielding 78 seedlings (χ^2 = 1.282, *P* = 0.258) (Table 8.3). Although White Chiffon® exhibits the *spotless* phenotype, these segregation tests confirm that it breeds like a heterozygote for the *geisha* trait once recessive epistasis of the *spotless* gene is released. To further confirm the heterozygous group, self-pollinations and intercrosses were performed within the group yielding a total of 167 seedlings for evaluation (Fig. 8.8). Two self-pollinations within the putative heterozygous group yielded the most progeny among all combinations. Self-pollinations of 'Woodbridge' yielded 47 progeny and a segregation ratio of 3:1 (colorful : *geisha*) (χ^2 = 0.007, *P* = 0.933). Self-pollinations of 'Aphrodite' yielded 44 progeny and a segregation ratio of 3:1 (colorful : *geisha*) (χ^2 = 1.939, *P* = 0.164) (Table 8.3).

Among the intercrosses in the heterozygous group, the cross Lavender Chiffon™ x White Chiffon® was most prolific, yielding 31 seedlings that segregated 3:1 (colorful : *geisha*). In addition, Strawberry Smoothie™, not originally included in the recessive *geisha* group due to a lack of self-pollinations and intercrosses within that group, was later confirmed by reciprocal crosses with Lavender Chiffon™ (Fig. 8.8) yielding 33 seedlings that segregated 1:1 (colorful : *geisha*). Self-pollinations of 'Woodbridge' yielded 47 progeny and a segregation ratio of 3:1 (colorful : *geisha*) (χ^2 = 0.273, *P* = 0.602).

Recessive testcrosses between the *geisha* group and the putative homozygous dominant group yielded segregation ratios of 1:0 (colorful : *geisha*) for all cross combinations (Table 8.3). Out of 1,083 progeny evaluated, only five seedlings were classified in the *geisha* phenotype category (Table 8.3). These seedlings were likely *spotless* flowers, rather than white flowers with eyespots, and mistakenly classified in the *geisha* phenotype category. The most prolific testcross was the reciprocal cross between Blue Chiffon™ and 'Red Heart', yielding 81 colorful progeny and no *geisha* phenotypes (Table 8.3). Another interesting cross was between Lil' Kim™ and 'Buddha Belly', resulting in a segregation ratio of 1:0 (colorful : *geisha*). This suggests that, although 'Buddha Belly' exhibits the *spotless* phenotype, it breeds like a homozygous dominant once recessive epistasis of the *spotless* gene is released. However, only seven seedlings from this cross were observed and the only other testcrosses with 'Buddha Belly' were performed with the homozygous dominant group. Therefore, further testcrosses will need to be performed to confirm this initial result.

To further confirm the putative homozygous dominant group, self-pollinations and intercrosses were performed (Fig. 8.8) resulting 167 seedlings segregating 1:0 (colorful : *geisha*) (Table 8.3). Only one seedling among this group was classified as a *geisha* phenotype, but was likely a *spotless* seedling incorrectly categorized as *geisha*. In yet another attempt to confirm the putative heterozygous dominant group, testcrosses were made to the putative heterozygous group, yielding 173 seedlings that segregated 1:0 (colorful : *spotless*). Only two seedlings were categorized as *geisha*, but were likely miscategorized *spotless* seedling.

Based on these combined results, we propose that the *geisha* phenotype (white to blush pink phenotype with an eyespot) is controlled by a single recessive allele (named *geisha*) with the colorful phenotype exhibiting incomplete dominance in the heterozygous taxa. Nearly all taxa in the homozygous dominant group were blue, with exception of 'Lucy' (a deep pink) and 'Buddha Belly' (a *spotless* white) (Fig. 8.8). Nearly all taxa in the heterozygous group were pink to lavender, with the exception of White Chiffon® (a *spotless* white) (Fig. 8.8). The true color (underlying genes for color in the petal body) of 'Buddha Belly' and White Chiffon® are likely masked by an epistatic interaction with the *spotless* allele. Although *H. syriacus* is reported to be a tetraploid, the *geisha* phenotype was observed to segregate according to simple Mendelian diploid inheritance patterns. Therefore, for simplicity, we propose the diploid genotype notation of *gg* to represent the *geisha* phenotype, and (*GG, Gg*) to represent the colorful phenotype. However, all of the petal body phenotypes are under the control of a recessive epistatic *spotless* gene.

One possible explanation for the segregation patterns of *spotless* and *geisha* phenotypes emerges when considering the flavonoid biosynthetic pathway (Petrussa et al., 2013) (Fig. 8.9). If the gene conferring the *spotless* phenotype lies upstream of the three pathways involved in flower color expression in *H. syriacus*, then the cyanidins responsible for the red eyespot, the peonidins and pelargonidins responsible for salmon-pink and blush pink color, and the delphinidins, petuniadins, and malvidins responsible for deep pink, lavender, and blue flowers will not be expressed (Fig. 8.9). In addition, if the gene conferring the *geisha* phenotype inhibits or down-regulates the delphinidin pathway, responsible for dark pink, lavender, and blue

pigments, then plants that only produce cyanidins, peonidins, and pelargonidins may make up the *geisha* phenotype (Fig. 8.9). Plants that only produce cyanidins, isolated to the eyespot region, would likely be white with a red eyespot. Plants that produce trace levels of cyanidin, peonidin and pelargonidin in the petal body may be responsible for the white/pink bicolor and blush-pink flowers seen in some of the *geisha* phenotypes (Fig. 8.9).

A previous study on extracted anthocyanins in *H. syriacus* lends evidence to this theory. Kim et al. (1989a) analyzed pigments from *H. syriacus* flowers exhibiting eyespots, with petal bodies including white (*geisha*), blush (*geisha*), dark pink, lavender, and blue. Total anthocyanins were significantly reduced in the white and blush flowers, with the majority of pigments in the petal body made up of cyanidin and pelargonidin (Kim et al., 1989a). Dark pink flowers (putative heterozygotes) produced the most total anthocyanins with a significant percent anthocyanins from all five categories and the lowest percent from pelargonidins (Kim et al., 1989a). The majority of pigments produced in lavender flowers were from the delphinidin pathway: delphinidin, petunidin, and malvidin (Kim et al., 1989a). The vast majority of pigments in blue flower were from the malvidin group, with lower percentages of delphinidin and petunidin compared to lavender flowers (Kim et al., 1989a).

To further confirm this theory of color segregation, self-pollinations and backcrosses were performed in an attempt to develop F_2 and backcross populations in a wide range of F_1 progeny from multiple cross combinations. However, growth was stunted in many of the F_2 and backcross progeny, likely due to inbreeding depression.

Therefore, data were collected only on two crosses that provided enough vigorous F_2 and backcross seedlings to perform segregation analysis on the *spotless* and *geisha* phenotypes. *Spotless* phenotypes were removed from the segregation tests on the *geisha* phenotype.

In the first cross, Lil' Kim™ (*SSgg*) x Blue Chiffon™ (*SsGG*), the F_1 population made up of 28 seedlings segregated 1:0 for both eyespot (eyespot : *spotless*) and for color (colorful : *geisha*) (Table 8.4). Based on the parent genotypes, F_1 seedling genotypes likely segregated 1:1 for (*SSGg : SsGg*). Due to time constraints and sizes of each F_1 seedling, individual plants were grouped together into genotype families based on the presence or absence of *spotless* phenotypes in their F_2 (S_1) progenies after self-pollinations. Therefore, the first F_2 family from self-pollinations of F_1 (*SSGg*) family segregated 1:0 (eyespot : *spotless*) and 3:1 (colorful : *geisha*) ($\chi^2 = 1.024$, $P = 0.312$). The second F_2 family from self-pollinations of F_1 (*SsGg*) family resulted in a segregation ratio of 3:1 (eyespot : *spotless*) ($\chi^2 = 2.232$, $P = 0.135$) and a segregation ratio of 3:1 (colorful : *geisha*) ($\chi^2 = 3.205$, $P = 0.073$) (Table 8.4).

Next, backcrosses of the F_2 families to both parents were attempted. The backcross of the F_1 (*SSGg*) family to Lil' Kim™ (*SSgg*) resulted in a segregation ratio of 1:0 (eyespot : *spotless*) and a segregation ratio of 1:1 (colorful : *geisha*) ($\chi^2 = 1.815$, $P = 0.178$) (Table 8.4). The backcross of the F_1 (*SsGg*) family to Lil' Kim™ (*SSgg*) resulted in a segregation ratio 1:0 (eyespot : *spotless*) and a segregation ratio of 1:1 (colorful : *geisha*) ($\chi^2 = 0.027$, $P = 0.869$) (Table 8.4). Backcrosses to Blue Chiffon™ (*SsGG*) were only successful with the F_1 (*SsGg*) family, yielding a

segregation ratio of 3:1 (eyespot : *spotless*) and segregation of 1:0 (colorful : *geisha*) (Table 8.4). Only three out of the 90 seedlings evaluated were scored as *geisha*, but were likely *spotless* mutants that were miscategorized (Table 8.4).

In the second cross, Fiji™ (*SSgg*) x White Chiffon® (*ssGg*), the F_1 population made up of 61 seedlings segregated 1:0 (eyespot : *spotless*) and 1:1 (colorful : *geisha*) ($\chi^2 = 1.984$, $P = 0.159$) (Table 8.5). Due to time constraints and sizes of each F_1 seedling, individual plants were grouped together into genotype families based on their petal body phenotype: F_1 (*Ssgg*) *geisha* family and F_1 (*SsGg*) colorful family (Table 8.5). Self-pollinations produced few progeny for segregation analysis (Table 8.5). However, the F_2 family resulting from self-pollinations of the F_1 (*Ssgg*) family resulted in a segregation ratio of 3:1 (eyespot : *spotless*) ($\chi^2 = 0.667$, $P = 0.414$) and a segregation ratio of 0:1 (colorful : *geisha*) (Table 8.5). The F_2 family resulting from self-pollinations of the F_1 (*SsGg*) family resulted in a segregation ratio of 3:1 (eyespot : *spotless*) ($\chi^2 = 0.095$, $P = 0.758$) and a segregation ratio of 3:1 (colorful : *geisha*) ($\chi^2 = 0.758$, $P = 0.384$). Next, backcrosses of the F_2 families to both parents were attempted. Backcross to Fiji™ failed to yield any progeny. However, both F_1 families produced progeny when backcrossed to White Chiffon® (Table 8.5). The backcross of the F_1 (*Ssgg*) family to White Chiffon® (*ssGg*) resulted in a segregation ratio 1:1 (eyespot : *spotless*) ($\chi^2 = 0.074$, $P = 0.785$) and a segregation ratio of 1:1 (colorful : *geisha*) ($\chi^2 = 0.000$, $P = 1.000$) (Table 8.5). The backcross of the F_1 (*SsGg*) to White Chiffon® (*ssGg*) resulted in only a few progeny for analyses; therefore, further segregation test will be necessary for this cross.

In the current study, most crosses among fully heterozygous taxa (*SsGg*) yielded too few seedlings to do a full segregation test for recessive epistasis of *spotless* over colorful and *geisha* phenotypes. However, self-pollinations of 'Woodbridge' yielded 66 seedlings with significant recessive epistatic segregation ratio of 9:3:4 (colorful : *geisha* : *spotless*) (χ^2 = 0.512, *P* = 0.774) (Table 8.6). In addition, self-pollinations of the heterozygous F_1 family (*SsGg*) produced from the cross Lil' Kim™ x Blue Chiffon™ yielded 79 seedlings with a significant recessive epistatic segregation ratio 9:3:4 (colorful : *geisha* : *spotless*) (χ^2 = 0.774, *P* = 0.056) (Table 8.6).

Results from crosses with blue-flowered taxa revealed an interesting segregation pattern. Hybrid seedlings exhibiting blue flowers were only recovered from crosses among blue flowered taxa. All other hybrid combinations with blue flowers resulted in pink to lavender flowers or *spotless* flowers in the F_1 seedlings. In addition, in the anthocyanin study by Kim et al. (1989a), blue flowers expressed a majority of pigments from the malvidin group of anthocyanins, with minimal amounts of delphinidin and petunidin. Therefore, another recessive gene downstream in the delphinidin biosynthesis pathway was hypothesized to effect the transition to pink or lavender flowers (petunidin) leaving only blue (malvidin) pigments in the homozygous recessive taxa carrying this hypothetical allele. CIE L* a* b* values for all self-pollinated and intercrossed taxa in the blue-flowered group were investigated, and sorted for their color components. Based on this set of blue progeny, we determined that a true blue flower exhibits a CIE L* <65, CIE a* < 18.3, and CIE b* < -18.3. This score was used to bin blue vs. pink-lavender flowers in F_1, F_2, and

backcross segregation tests for a recessive blue allele in the cross Lil Kim™ x Blue Chiffon™ (Table 8.7). *Spotless* phenotypes were removed from the segregation analyses.

A total of 52 (S_1) seedlings resulting from self-pollinations of 'Blue Bird', Blue Chiffon™, and Blue Satin® yielded segregation ratios of 0:1 (pink/lavender : blue) (Table 8.7). In addition, a total of 19 F_1 seedlings from the cross Blue Satin® x 'Bluebird' resulted in a segregation ratio of 0:1 (pink/lavender : blue) (Table 8.7). For the cross Lil' Kim™ x Blue Chiffon™, we observed a segregation ratio of 1:0 (pink/lavender : blue) in the F_1 population (Table 8.7). Self-pollination of the F_1 family produced an F_2 population that segregated almost entirely pink/lavender after 93 observed seedlings, deviating significantly from the expected 3:1 segregation ratio (Table 8.7). Backcrosses to Blue Chiffon™ yielded slightly more blue progeny (eight blue progeny out of the 87 observed), but progeny deviated significantly from the expected 1:1 segregation ratio (Table 8.7). When CIEL*a*b* estimates from the backcross progeny were compared to the blue progeny resulting from blue-flowered self-pollinations and blue-flowered intercrosses, the wide segregation of blue to pink/lavender color in the backcross progeny becomes obvious (Fig. 8.10). This could be due to the fact that delphinidin (the precursor to petunidin and malvidin) is a stable pigment and the interplay between these three types of anthocyanins makes it difficult to delineate a true blue pigment (Fig. 8.1). In addition, the blue trait in *H. syriacus* could simply be controlled by multiple genes at different loci.

Depth of color. Although different categories may be under control of simple genes that segregate in Mendelian inheritance patterns, there was a wide range of total

anthocyanin production in progeny not exhibiting the *spotless* and *geisha* phenotype. Categorizing average flower colors (of colorful flowers) from specific combinations of elite cultivars may provide information on specific crosses useful for enriching breeding populations for colorful flowers. In the current study, the best measure of depth of color (irrespective of hue) was the CIE L* value, with the lower CIE L* indicating deeper colors, likely with more pigment production.

Individual cross combinations had a significant effect ($P < 0.0001$) on average CIE L* value among seedlings when *geisha* as *spotless* phenotypes were removed (Table 8.8). The deepest average pigments were produced with hybrids between dark pink and blue-flowered taxa, including the cross between tetraploid, blue-flowered Blue Satin® and the hexaploid, pink-flowered 'Pink Giant' (Fig. 8.11) with an average CIE L* of 52.6 ± 1.6 (Table 8.8). This may indicate that increased ploidy level could result in darker flower pigments in *H. syriacus*. Equally dark-flowered taxa were recovered from self-pollinations of Blue Satin® (53.4 ± 1.0), self-pollinations 'Blue Bird' (55.5 ± 0.9), reciprocal crosses between Blue Satin® and 'Minerva' (55.5 ± 1.4), and the cross 'Minerva' x 'Blue Bird' (55.5 ± 1.4) (Table 8.8). Progeny with some of the lightest average pigments (high CIE L*) were observed from crosses between the blue-flowered taxon Blue Chiffon™ and the *geisha* taxa 'Diana', 'Red Heart', and Lil' Kim™ at CIE L* values of 68.3 ± 0.4, 67.9 ± 0.04, 67.8 ± 0.5, respectively (Table 8.8). Conspicuously, some of the darkest average flower pigments were made up from crosses between homozygous dominant parents for *geisha* (*GG* x *GG*), and crosses with heterozygotes (*GG* x *Gg*). Conversely, some of the lightest observed pigments were observed in crosses between

geisha phenotypes and heterozygotes (*Gg* x *gg*). Therefore, quantitative improvement on color depth may be possible by eliminating the *geisha* allele from a breeding population and making selections in each generation for low CIE L* values. In addition, *spotless* alleles would also have to be removed from the breeding population as not to waste time and space, especially in the blue-flowered taxa. In the current study, all blue-flowered taxa carried the *spotless* allele, and it remains unclear if there are any true-breeding (*SSGG*) blue taxa available for breeders of *H. syriacus*. Future breeding efforts will focus on crossing blue-flowered seedlings to recessive testers ('Diana', White Chiffon®, 'Buddha Belly') to develop a true-breeding blue *H. syriacus*.

Petal number. The number of petals (five true petals + petaloid stamen) was found to vary significantly by cross ($P < 0.0001$) and by cross type ($P < 0.0001$) including single- x single-flowered reciprocal crosses (S | S), single- x double-flowered reciprocal crosses (S | D), and double- x double-flowered reciprocal crosses (Table 8.9). Observations of petal number segregation among these cross types found a relatively consistent trend, where (S | S) cross types yielded mostly single-flowered progeny with five true petals and a few petaloid stamens on the monadelphus column (Fig. 8.12A). However, (S | D) and (D | D) cross types yielded progeny with a continuous distribution between single and double phenotypes (Fig. 8.12B-D). We also observed that the majority of this quantitative distribution usually fell between the petal counts of the two parents, with some transgressive segregants found outside the extremes of the petal number range. Therefore, quantitative improvement on petal number may be possible by selecting and recombining progeny with heavy petal

production over successive generations. In the current study, the highest average petal numbers across all cross types were found in (D | D) crosses at 36.0 ± 2.4 petals, followed by (S | D) crosses at 13.9 ± 1.0 petals, and (S | S) crosses with 5.9 ± 0.2 petals (Table 8.9). Within the (D | D) crosses, the individual crosses that yielded the highest average petal count were obtained from crosses performed among the Chiffon® series and Smoothie™ series (Fig. 8.13A-B). The two crosses in the current study that produced highest average petal number were self-pollinations of Pink Chiffon® at 52.9 ± 1.6 petals and the cross White Chiffon® x Strawberry Smoothie™ at 51.3 ± 3.0 petals (Table 8.9).

Another observation on double-flower production is the occasional presence of fully double flowers in (S | D) and (D | D) crosses. One parent, 'Blushing Bride', not only produced petaloid stamens, but much of the monadelphus column and sometimes the entire pistil become petaloid. Flowers of 'Blushing Bride' that did produce functional stigmas (Fig. 8.5B) were used in crosses, and most of the resulting progeny were found to exhibit flowers with all whorls converted to petals (Fig. 8.14A-C). Although these flowers are a dead end for breeding, they represent novel floral phenotypes that can be combined with other novel traits such as bicolor petal in crosses with the *geisha* taxa Fiji™ (Fig. 8.14B) and polyploidy in crosses with the hexaploid 'Pink Giant' (Fig. 8.14C).

Flower size. Flower size, as measured by petal area (length x width), varied significantly among cross combinations ($P < 0.0001$) and cross type (S | S), (S | D), and (D | D) in *H. syriacus* ($P = 0.0020$). However, of all floral traits measured, flower size was observed to vary the most with environment (J. Lattier – Personal

Observation). As plants were not completely randomized by year and environment, we recommend caution in interpreting flower size estimates. In the current study, flower size of progeny resulting from (S | D) cross types was found to be significantly larger than both (S | S) and (D | D) (Table 8.10). Double-flowering (petaloid stamen) has shown in previous research to be triggered by genes controlling laminar growth, particularly MADS-box genes (Almeida et al., 2013). B-class and C-class gene expression have been shown to expand beyond the tradition ABC model in some plants (Almeida et al., 2013). Perhaps a concomitant upregulation or expression of genes controlling laminar growth in stamen not only result in petaloid stamen, but may also result in increased laminar growth in the true petals, resulting in wider, overlapping petals in F_1 hybrids of (S | D) crosses in *H. syriacus*. Many of the F_1 seedlings from (S | D) were observed to have wide, overlapping petals, adding to their petal area estimates (Fig. 8.15A). Unlike the true petals in many (D | D) crosses, there did not appear to be any loss in petal length in F_1 progeny of (S | D) seedlings, with some of the widest overall flowers discovered from this cross type (Fig. 8.15B).

Other phenotypic observations. Within the *geisha* phenotype group, one taxa, Fiji™, was found to exhibit bicolor petals, with pink or sometimes red pigment (reminiscent of the eyespot) developing on the abaxial side of mature buds. This unusual and attractive phenotype was heritable in F_1 crosses with Fiji™; the bicolor phenotype was most easily identified in mature, expanding buds (Fig. 8.16A). The exact source of this pigment remains unclear. A similar taxon, 'Elegantissimus' (a.k.a. 'Lady Stanley'), was used to investigate anthocyanin composition in petal tissues in a previous study (Kim et al., 1989a). This study found that the majority of

pigment in the petal body was composed of pelargonidins, with a lower, but significant, percent of cyanidins present. However, the expression of this trait was variable, with full-sib progeny exhibiting a color range from conspicuous bicolor buds (Fig. 8.16A) to blush-pink buds (Fig. 8.16B). When flower buds opened, blush-pink buds often appear white while bicolor buds open with a bicolor combination of white and pink (Fig. 8.16C) which is most visible on the abaxial surface of the petal (Fig. 8.16D).

Although the majority of plants exhibited stable flower phenotypes, bud sport mutations are inevitable when observing thousands of branches upon thousands of hybrid seedlings. However, we observed only two interesting sport branches throughout our study. The first was a pink-flowered hybrid that produced a *geisha* branch that was white with an eyespot (Fig. 8.17A). The second was a branch sport on an F_1 hybrid of Minerva x Blue Satin® that produced blush pigment in the petal body, but lacked an eyespot (Fig. 8.17B). The latter sport branch was propagated and stability of this trait will be evaluated over subsequent years. If stable, the testcrosses will be performed to investigate the heritability of this trait. If the *spotless* and *geisha* phenotypes arose as chance mutations and have proven heritable, perhaps this novel flower form will perform similarly in testcrosses. If pigment production in the petal body can be bred in the absence of an eyespot, this discovery could lead to new breeding objectives in *H. syriacus* and will likely lead to novel flower forms in the years to come.

Another interesting phenotype observed in the breeding collection was the increased stem and node size in ‘Buddha Belly’. This rare cultivar was acquired to

provide another recessive tester for the *spotless* phenotype when discovering variable cross- and self-incompatibility among cultivars in a previous fertility study (Chapter X). The majority of the F_1 seedlings from crosses with 'Buddha Belly' exhibited this novel stem phenotype, resulting in seedlings with stiffer and more upright stems when compared to other F_1 hybrids (Fig. 8.18). Before acquiring 'Buddha Belly', we observed that plants with weak, thin branches were heavily damaged in winter under snow load. Perhaps the 'Buddha Belly' phenotype will result in increased wood density and tensile strength of young branches, making them hardier to winter conditions. In addition, we observed no obvious reduction in vigor in F_1 hybrids of 'Buddha Belly' exhibiting thicker stems. Therefore, this phenotype may provide useful material for creating container plant forms, such as standards. Future efforts will be undertaken to determine the inheritance of this trait and its utility in a breeding program for *H. syriacus*.

The current study represents a comprehensive investigation into segregation of floral traits, including simply inherited traits such as eyespot and petal body color, and quantitative traits such as depth of color, flower size, and petal number. Genotype data for *spotless* and *geisha* traits, and phenotype data for color is presented for parent taxa in this study (Table 8.11). This information will aid future breeders of and further future research into the heritability of traits in *H. syriacus*.

Literature Cited

Almeida, A.M.R., A. Brown, and C.D. Specht. 2013. Tracking the development of the petaloid fertile stamen in *Canna indica*: insights into the origin of androecial petaloidy in the Zingiberales. AoB Plants 5: DOI: 10.1093/aobpla/plt009

Bae, S.H., A. Younis, Y.-J. Hwang, and K.-B. Lim. 2015. Various pollen morphology in *Hibiscus syriacus*. Flower Res. J. 23:125-130.

Bai, X.D., Z.G. Cao, Y. Wang, Z.H. Yu, X.F. Zhang, and C.N. Li. 2013. Crop segmentation from images by morphology modeling in the CIE L*a*b* color space. Computers and Electronics in Agriculture 99: 21-34.

Bintory, M.A., G.K. Seetharamu, P.M. Munikrishnappa, G.K. Ramegowda, and G. Basavaraj. 2015. Evaluation of the colour of dried dutch rose flowers using a colorimeter. J. Hort 2: http://dx.doi.org/10.4172/2376-0354.1000157.

Contreras, R. and J.D. Lattier. 2014. Targeted breeding of rose-of-sharon to improve a garden classic. Digger (August 2014):47-50.

Contreras, R.N., J.M. Ruter, and W.W. Hanna. 2009. An Oryzalin-induced autoallooctaploid of *Hibiscus acetosella* 'Panama Red". J. Amer. Soc. Hort. Sci. 134:553-559.

Dirr, M.A. Manual of Woody Landscape Plants: Their Identification, Ornamental Characteristics, Culture, Propagation, and Uses. 6th Ed. Champaign, Illinois: Stipes Publishing LLC, 2009.

García-Marino, M., M.L. Escudero-Gilete, F.J. Heredia, M.T. Escribano-Bailón, and J.C. Rivas-Ganzalo. 2013. Color-copigmentation study by tristimulus colorimetry (CIELAB) in red wines obtained from Tempranillo and Graciano varieties. Food Res. Intl. 51:123-131.

García-Marino, M., M.L. Escudero-Gilete, M.T. Escribano-Bailón, M.L. Gonzalez-Miret, J.C. Rivas-Ganzalo, and F.J. Heredia. 2012. Colorimetric characteristics of phenolic fractions obtained from Tempranillo and Graciano wines through the use of different instrumental techniques. Analytica Chimica Acta 732: 153-161.

Gettys, L.A. 2012. Genetic control of white flower color in scarlet rosemallow (*Hibiscus coccineus* Walter). J. Hered. 103:594-597.

Gonnet, J.-F. 1993. CIELab measurement, a precise communication in flower colour: An example with carnation *(Dianthus caryophyllus*) cultivars. J. Hort. Sci. 68: 499-510.

Gonnet, J.-F. 1995. A colorimetric look at the RHS Chart – Perspectives for an instrumental determination of colour codes. J. Hort. Sci. 70:191-206.

Griesbach, R.J. 1996. The inheritance of flower color in *Petunia* hybrid Vilm. J. Hered. 87:241-245.

Ha, Y.-M., D.Y. Kim, and I.S. Han. 2010. A new cultivar ‘Daemang’ with long red eye spot and large flower by interspecific cross of *Hibiscus* species. Korean J. Sci. Technol. 28:711-714.

Ha, Y.-M., K.-B. Lim, and K.-K. Shim. 2015. Development of a new *Hibiscus* cultivar 'Daewangchun' with vigorous growth and unique red eye through interspecific hybridization. Korean J. Hort. Sci. Technol. 33:453-458.

Ha, Y.-M., K.-K. Shim, H.-C. Kang, and K.-B. Lim. 2014. A new cultivar 'Tohagol Red' with unique flower shape and color through interspecific hybridization of *Hibiscus* species. Flower Res. J. 22:278-282.

Kim, J.H., H. Okubo, K. Fujieda, and S. Uemoto. 1989a. Anthocyanin pigmentation patterning in petals of *Hibiscus syriacus*. J. Faculty Agr. Kyushu Univ. 33:253-258.

Kim, J.H., H. Okubo, K. Fujieda, and S. Uemoto. 1989b. Changes of petal colors during senescence in *Hibiscus syriacus*. J. Faculty Agr. Kyushu Univ. 33:259-256.

Koski, M.H. and T.-L. Ashman. 2013. Quantitative variation, heritability, and trait correlations for ultraviolet traits in *Argentina anserina* (Rosaceae): Implications for floral evolution. Intl. J. Plant Sci. 174:1109-1120.

Menzel, M.Y. and E.D. Wilson. 1969. Genetic relationships in *Hibiscus* sect. *Furcaria*. Brittonia 21:91-125.

Okino, E.Y.A., T.C.M. Pastore, J.A.A. Camargos, V.T.R. Coradin, D.E. Teixeira, M.A.E. Santana and C.W. Fagg. 2015. Accelerated laboratory test of three Amazonian wood specie called Tauari, exposed to white- and brown-rot fungi and color response according to the CIE L* A* B* system. Ciência Florestal 25:581-593.

Owen, C.R., and H.D. Bradshaw. 2011. Induced mutations affecting pollinator choice in *Mimulus lewisii* (Phrymaceae). Arthropod Plant Interactions 5:235-244.

Petrussa, E., E. Braidot, M. Zancani, C. Peresson, A. Bertolini, S. Patui, and A. Vianello. 2013. Plant flavonoids – Biosynthesis, transport and involvement in stress responses. Intl. J. Mol. Sci. 14: 14950-14973. doi:10.3390/ijms140714950.

Royal Horticulture Society. 2007. The Royal Horticulture Society Colour Chart, 5th ed. London, England.

Satya, P., M. Karan, D. Sarkar, and M.K. Sinha. 2012. Genome synteny and evolution of AABB allotetraploids in *Hibiscus* section *Furcaria* revealed by interspecific hybridization, ISSR and SSR markers. Plant Systematics Evolution 298:1257-1270.

Skovsted, A. 1941. Chromosome numbers in the Malvaceae II. Comptes rendus des traveaux du laboratoire Carlberg. Série Physiologique 23:195-242.

Syafaruddin, K. Kobayashi, Y. Yoshioka, A. Horisaki, S. Niikura, and R. Ohsawa. 2006. Estimation of heritability of the nectar guide of flowers in *Brassica rapa* L. Breeding Sci. 56:75-79.

Van Laere, K. 2008. Interspecific Hybridisation in Woody Ornamentals. Ghent University. Faculty of Bioscience Engineering, Ghent, Belgium.

Wilson, E.D. 1994. The genome biogeography of *Hibiscus* L. section *Furcaria* DC. Genet. Res. Crop Evolution 41:13-25.

Wilson, E.D. 1999. Revision of *Hibiscus* section *Furcaria* (Malvaceae) in Africa and Asia. Bul. Natural History Museum London (Bot.) 29:47-79.

Yuan, Y.-W., J.M. Sagawa, V.S. Di Stilio, and H.D. Bradshaw, Jr. 2013. Bulk segregant analysis of an induced floral mutant identifies a *MIXTA*-Like R2R3 *MYB* controlling nectar guide formation in *Mimulus lewisii*. Genetics 194:523-528.

Yue, B., B.A. Vick, W. Yuan and J. Hu. 2008. Mapping of the 2 genes controlling lemon ray flower color in sunflower (*Helianthus annuus* L.). J. Hered. 99:564-567.

Tables

Table 8.1. Source material for *Hibiscus syriacus* L. breeding in the Contreras Lab at Oregon State University.

Cultivar[z]	Trade name[y]	Accession no.[x]	Source[w]
'American Irene Scott'	Sugar Tip®	12-0019	Bailey Nurseries
'Antong Two'	Lil' Kim™	12-0021	Bailey Nurseries
'Aphrodite'		13-0054	Monrovia
		11-0215	Bailey Nurseries
'Ardens'		13-0050	Blue Heron
'Blue Bird'		11-0219	Monrovia
		13-0057	Monrovia
'Blushing Bride'		13-0048	Blue Heron
		13-0059	Monrovia
'Bricutts'	China Chiffon™	13-0060	Monrovia
'Buddha Belly'		14-0128	Yamaguchi Nursery
'Collie Mullins'		13-0061	Monrovia
'Diana'		13-0062	Monrovia
		11-0211	Bailey Nurseries
'DS01BS'	Blueberry Smoothie™	14-0092	Greenleaf Nursery
'DS02SS'	Strawberry Smoothie™	14-0091	Greenleaf Nursery
'DS03RS'	Raspberry Smoothie™	14-0094	Greenleaf Nursery
'DS04PS'	Peppermint Smoothie™	14-0093	Greenleaf Nursery
'DVPazurri'	Azurri Satin®	13-0055	Monrovia
		14-0188	Spring Meadow
		16-0015	Forestfarm
'Floru'	Violet Satin®	13-0118	JC Raulston Arboretum
		13-0119	JC Raulston Arboretum
'Helene'		13-0063	Monrovia
		13-0116	JC Raulston Arboretum
		13-0117	JC Raulston Arboretum
'JWNfour'	Pink Chiffon®	13-0067	Monrovia
'Lucy'		11-0216	Bailey Nurseries
'Marina'	Blue Satin®	13-0094	JC Raulston Arboretum
		11-0210	Bailey's Nursery
'Mathilde'	Blush Satin®	13-0058	Monrovia
'Mineru'	First Editions® Tahiti™	12-0024	Bailey Nurseries
		13-0098	Bailey Nurseries
'Minerva'		13-0051	Blue Heron
		13-0066	Monrovia
		11-0213	Bailey Nurseries

Table 8.1 (continued). Source material for *Hibiscus syriacus* L. breeding in the Contreras Lab at Oregon State University.

Cultivar[z]	Trade name[y]	Accession no.[x]	Source[w]
'Minfren'	First Editions® Bali™	12-0023	Bailey Nurseries
'Minrosa'	Rose Satin®	13-0068	Monrovia
		13-0068	Monrovia
'Minspot'	First Editions® Fiji™	12-0022	Bailey Nurseries
'Minsygrbl1'	First Editions® Hawaii™	12-0020	Bailey Nurseries
		13-0096	Bailey Nurseries
'Notwoodone'	Lavender Chiffon™	13-0046	Blue Heron
		13-0064	Monrovia
'Notwoodthree'	Blue Chiffon™	13-0056	Monrovia
		11-0218	Blue Heron
'Notwoodtwo'	White Chiffon®	13-0044	Blue Heron
'Pink Giant'		11-0217	Bailey Nurseries
'Red Heart'		13-0049	Blue Heron
'Woodbridge'		11-0214	Bailey Nurseries
		13-0047	Blue Heron

[z]Cultivar name.

[y]Trademark name.

[x]Accession number in research collection at the Ornamental Plant Breeding Lab, Oregon State University, Corvallis, OR.

[w]Container plant collected from the following sources: Bailey Nurseries, Yamhill, OR; Blue Heron Farm, Corvallis, OR; Forestfarm Nursery, Williams, OR; Greenleaf Nursery, Grants Pass, OR; JC Raulston Arboretum, Raleigh, NC; Monrovia, Dayton, OR; Spring Meadow Nursery, Grand Haven, MI; Yamaguchi Plantsman Nursery, Gifu, Japan.

Table 8.2. Segregation of *spotless* phenotype in F_1 and S_1 progeny of *Hibiscus syriacus*.

Putative genotypes[z]	Parent 1	Parent 2	Eyespot[y]	*Spotless*[x]	Total[w]	Expected ratio[y]	*P*	χ^2
ss | *ss*								
	'Buddha Belly'	'Diana'	0	75	75	0:1	--	--
	'Buddha Belly'	White Chiffon®	0	26	26	0:1	--	--
	'Diana'	White Chiffon®	0	74	74	0:1	--	--
	White Chiffon®	White Chiffon®	0	71	71	0:1	--	--
ss | *Ss*								
	'Blue Bird'	'Diana'	4	3	7	1:1	0.705	0.143
	'Blue Bird'	'Buddha Belly'	6	2	8	1:1	0.157	2.000
	Blue Chiffon™	'Diana'	49	63	112	1:1	0.186	1.750
	Blue Chiffon™	White Chiffon®	5	10	15	1:1	0.197	1.667
	Blue Satin®	'Buddha Belly'	81	87	168	1:1	0.643	0.214
	Blue Satin®	'Diana'	47	26	73	1:1	0.014	6.041
	Blue Satin®	White Chiffon™	57	54	111	1:1	0.776	0.081
	'Buddha Belly'	Blue Chiffon™	5	4	9	1:1	0.739	0.111
	'Diana'	'Minerva'	6	5	11	1:1	0.763	0.093
	'Diana'	'Red Heart'	52	52	104	1:1	1.000	0.000
	'Diana'	'Woodbridge'	20	16	36	1:1	0.505	0.444
	'Woodbridge'	White Chiffon®	2	3	5	1:1	0.655	0.200
ss | *SS*								
	'Aphrodite'	'Diana'	81	0	81	1:0	--	--
	Bali™	'Diana'	45	0	45	1:0	--	--
	'Buddha Belly'	Lil' Kim™	7	0	7	1:0	--	--
	'Diana'	China Chiffon™	4	0	4	1:0	--	--
	'Diana'	Fiji™	13	0	13	1:0	--	--
	'Diana'	Lil' Kim™	14	0	14	1:0	--	--
	Fiji™	White Chiffon®	77	0	77	1:0	--	--
	Lavender Chiffon™	White Chiffon®	31	0	31	1:0	--	--
	Lil' Kim™	White Chiffon®	73	0	73	1:0	--	--
	'Lucy'	'Diana'	6	0	6	1:0	--	--
	Pink Chiffon®	White Chiffon®	78	0	78	1:0	--	--
	White Chiffon®	Strawberry Smoothie™	19	0	19	1:0	--	--

Table 8.2 (continued). Segregation of *spotless* phenotype in F_1 and S_1 progeny of *Hibiscus syriacus*.

Putative genotypes[z]	Parent 1	Parent 2	Eyespot[y]	*Spotless*[x]	Total[w]	Expected ratio[v]	*P*	χ^2
Ss | *Ss*								
	'Blue Bird'	'Blue Bird'	26	11	37	3:1	0.506	0.441
	'Blue Bird'	'Minerva'	11	2	13	3:1	0.423	0.641
	Blue Chiffon™	'Red Heart'	85	23	108	3:1	0.374	0.790
	Blue Satin®	'Blue Bird'	19	3	22	3:1	0.218	1.515
	Blue Satin®	Blue Satin®	27	6	33	3:1	0.366	0.818
	Blue Satin®	'Minerva'	18	4	22	3:1	0.460	0.545
	Blue Satin®	'Red Heart'	24	9	33	3:1	0.763	0.091
	Blue Satin®	'Woodbridge'	17	2	19	3:1	0.145	2.123
	'Minerva'	'Minerva'	4	1	5	3:1	0.796	0.067
	'Red Heart'	'Red Heart'	8	5	13	3:1	0.262	1.256
	'Red Heart'	'Woodbridge'	12	7	19	3:1	0.233	1.421
	'Woodbridge'	'Minerva'	13	6	19	3:1	0.508	0.439
	'Woodbridge'	'Woodbridge'	49	19	66	3:1	0.575	0.314
	Hawaii™	Hawaii™	4	1	5	3:1	0.796	0.067
SS | *SS*								
	'Aphrodite'	'Aphrodite'	44	0	44	1:0	--	--
	'Aphrodite'	Bali™	66	0	66	1:0	--	--
	'Aphrodite'	Fiji™	14	0	14	1:0	--	--
	'Blushing Bride'	Fiji™	22	0	22	1:0	--	--
	Fiji™	Fiji™	16	0	16	1:0	--	--
	Lavender Chiffon™	Pink Chiffon®	12	0	12	1:0	--	--
	Lavender Chiffon™	Strawberry Smoothie™	33	0	33	1:0	--	--
	Pink Chiffon®	Pink Chiffon®	85	0	85	1:0	--	--
SS | *Ss*								
	'Aphrodite'	'Minerva'	17	0	17	1:0	--	--
	'Aphrodite'	'Red Heart'	56	0	56	1:0	--	--
	Bali™	'Blue Bird'	78	0	78	1:0	--	--
	'Blue Bird'	Lil' Kim™	45	1	46	1:0	--	--
	Blue Chiffon®	Fiji™	31	0	31	1:0	--	--
	Blue Satin®	Lil' Kim™	9	0	9	1:0	--	--
	'Blue Bird'	China Chiffon™	48	0	48	1:0	--	--
	'Blushing Bride'	Blue Chiffon™	32	0	32	1:0	--	--
	'Blushing Bride'	'Minerva'	4	0	4	1:0	--	--
	China Chiffon™	Blue Chiffon™	30	0	30	1:0	--	--
	Fiji™	Hawaii™	32	0	32	1:0	--	--
	Fiji™	'Woodbridge'	6	0	6	1:0	--	--
	Lil' Kim™	Blue Chiffon™	69	0	69	1:0	--	--
	Lil' Kim™	'Woodbridge'	21	0	21	1:0	--	--

Table 8.2 (continued). Segregation of *spotless* phenotype in F_1 and S_1 progeny of *Hibiscus syriacus*.

Putative genotypes[z]	Parent 1	Parent 2	Eyespot[y]	*Spotless*[x]	Total[w]	Expected ratio[v]	*P*	χ^2
???								
	'Aphrodite'	'Woodbridge'	15	1	16	1:0		
	'Aphrodite'	Blue Satin®	27	2	29	1:0		
	'Aphrodite'	'Blue Bird'	35	3	38	1:0		
	'Lucy'	'Red Heart'	15	4	19	3:1		

[z]Putative genotypes in reciprocal (" | ") combinations for the hypothetical, recessive *spotless* allele. Homozygous recessive = *ss*. Heterozygous = *Ss*. Homozygous dominant = *SS*. ??? = Unknown segregation pattern.

[y]Number of progeny exhibiting a red eyespot.

[x]Number of progeny lacking an eyespot

[w]Total number of progeny

[v]Expected segregation ratio of eyespot to *spotless* progeny.

Table 8.3. Segregation of *geisha* phenotype in F_1 and S_1 progeny of *Hibiscus syriacus*.

Putative genotypes[z]	Parent 1	Parent 2	Colorful[y]	*Geisha*[x]	Total[w]	Expected ratio[v]	*P*	χ^2
gg | gg								
	Bali™	'Diana'	0	45	45	0:1	--	--
	'Blushing Bride'	Fiji™	0	22	22	0:1	--	--
	'Diana'	Fiji™	0	13	13	0:1	--	--
	'Diana'	Lil' Kim™	0	14	14	0:1	--	--
	'Diana'	'Red Heart'	0	52	52	0:1	--	--
	Fiji™	Fiji™	0	6	6	0:1	--	--
	'Helene'	'Diana'	0	3	3	0:1	--	--
	Pink Chiffon®	'Diana'	0	5	5	0:1	--	--
	Pink Chiffon®	Pink Chiffon®	0	85	85	0:1	--	--
	'Red Heart'	'Red Heart'	0	8	8	0:1	--	--
gg | Gg								
	'Aphrodite'	'Diana'	39	42	81	1:1	0.739	0.111
	'Aphrodite'	Fiji™	7	7	14	1:1	1.000	0.000
	'Blushing Bride'	'Minerva'	2	2	4	1:1	1.000	0.000
	Fiji™	White Chiffon®	25	36	61	1:1	0.159	1.984
	Fiji™	'Woodbridge'	4	2	6	1:1	0.414	0.667
	Lavender Chiffon™	Pink Chiffon®	6	6	12	1:1	1.000	0.000
	Lavender Chiffon™	Strawberry Smoothie™	18	15	33	1:1	0.602	0.273
	Lil' Kim™	White Chiffon®	37	35	72	1:1	0.814	0.056
	Lil' Kim™	'Woodbridge'	12	9	21	1:1	0.513	0.429
	Pink Chiffon®	White Chiffon®	44	34	78	1:1	0.258	1.282
	Red Heart	'Woodbridge'	7	8	15	1:1	0.796	0.067
gg | GG								
	Bali™	'Blue Bird'	76	1	77	1:0	--	--
	'Blue Bird'	China Chiffon™	48	0	48	1:0	--	--
	'Blue Bird'	'Diana'	4	0	4	1:0	--	--
	'Blue Bird'	Lil' Kim™	44	0	44	1:0	--	--
	Blue Chiffon™	'Diana'	49	3	52	1:0	--	--
	Blue Chiffon™	Fiji™	26	0	26	1:0	--	--
	Blue Chiffon™	'Red Heart'	84	0	84	1:0	--	--
	Blue Satin®	'Diana'	47	1	48	1:0	--	--
	Blue Satin®	Lil' Kim™	9	0	9	1:0	--	--
	Blue Satin®	'Red Heart'	24	0	24	1:0	--	--
	'Blushing Bride'	Blue Chiffon™	32	0	32	1:0	--	--
	'Buddha Belly'	Lil' Kim™	7	0	7	1:0	--	--

Table 8.3 (continued). Segregation of *geisha* phenotype in F_1 and S_1 progeny of *Hibiscus syriacus*.

Putative genotypes[z]	Parent 1	Parent 2	Colorful[y]	*Geisha*[x]	Total[w]	Expected ratio[v]	*P*	χ^2
gg | GG (continued)								
	China Chiffon™	Blue Chiffon™	30	0	30	1:0	--	--
	Hawaii™	Fiji™	31	0	31	1:0	--	--
	Lil' Kim™	Blue Chiffon™	28	0	28	1:0	--	--
	'Lucy'	'Diana'	6	0	6	1:0	--	--
	'Lucy'	'Red Heart'	15	0	15	1:0	--	--
Gg | Gg								
	'Aphrodite'	'Aphrodite'	37	7	44	3:1	0.164	1.939
	'Aphrodite'	'Minerva'	13	4	17	3:1	0.889	0.020
	'Aphrodite'	'Woodbridge'	13	2	15	3:1	0.297	1.089
	Lavender Chiffon™	White Chiffon®	22	9	31	3:1	0.604	0.269
	'Woodbridge'	'Minerva'	11	2	13	3:1	0.423	0.641
	'Woodbridge'	'Woodbridge'	35	12	47	3:1	0.933	0.007
GG | GG								
	'Blue Bird'	'Blue Bird	31	0	31	1:0	--	--
	'Blue Bird'	Blue Satin®	19	0	19	1:0	--	--
	Blue Chiffon™	Blue Chiffon™	2	0	2	1:0	--	--
	Blue Satin®	Blue Satin®	22	0	22	1:0	--	--
	Blue Satin®	'Buddha Belly'	81	1	82	1:0	--	--
	'Blue Bird'	'Buddha Belly'	6	0	6	1:0	--	--
	'Buddha Belly'	Blue Chiffon™	5	0	5	1:0	--	--
GG | Gg								
	'Aphrodite'	'Blue Bird'	35	1	36	1:0	--	--
	'Aphrodite'	Blue Satin®	27	0	27	1:0	--	--
	Blue Chiffon™	White Chiffon®	5	0	5	1:0	--	--
	Blue Satin®	'Minerva'	18	0	18	1:0	--	--
	Blue Satin®	White Chiffon®	56	0	56	1:0	--	--
	Blue Satin®	'Woodbridge'	16	1	17	1:0	--	--
	'Minerva'	'Blue Bird'	11	0	11	1:0	--	--
	'Red Heart'	'Blue Bird'	3	0	3	1:0	--	--

Table 8.3 (continued). Segregation of *geisha* phenotype in F_1 and S_1 progeny of *Hibiscus syriacus*.

Putative genotypes[z]	Parent 1	Parent 2	Colorful[y]	*Geisha*[x]	Total[w]	Expected ratio[v]	*P*	χ^2
???								
	'Aphrodite'	Bali™	66	0	66			
	'Aphrodite'	'Red Heart'	41	15	56			
	'Diana'	China Chiffon™	1	3	4			
	'Diana'	'Minerva'	1	5	6			
	'Diana'	'Woodbridge'	7	13	20			
	White Chiffon®	Strawberry Smoothie™	6	13	19			

[z]Putative genotypes in reciprocal (" | ") combinations for the hypothetical, recessive *geisha* allele. Homozygous recessive = *gg*. Heterozygous = *Gg*. Homozygous dominant = *GG*. ??? = Unknown segregation pattern.

[y]Number of progeny exhibiting a red eyespot.

[x]Number of progeny lacking an eyespot

[w]Total number of progeny

[v]Expected segregation ratio of eyespot to *spotless* progeny.

Table 8.4. Segregation of *spotless* and *geisha* phenotypes in F_1, F_2, and backcross generations from the cross *Hibiscus syriacus* Lil' Kim™ × *H. syriacus* Blue Chiffon™.

Family[z]	Parent 1[y]	Parent 2[x]	Eyespot[w]	*Spotless*[v]	Total[u]	Segregation[t]	*P*	χ^2	Colorful[s]	*Geisha*[r]	Total[q]	Segregation[p]	*P*	χ^2
F_1														
	Lil' Kim™ (*SSgg*)	Blue Chiffon™ (*SsGG*)	28	0	28	1:0	--	--	28	0	28	1:0	--	--
F_2														
	F_1 (*SSGg*)	F_1 (*SSGg*)	55	0	55	1:0	--	--	38	17	55	3:1	0.312	1.024
	F_1 (*SsGg*)	F_1 (*SsGg*)	65	14	79	3:1	0.135	2.232	55	10	65	3:1	0.073	3.205
$BC_{(P1)}$														
	F_1 (*SSGg*)	Lil' Kim™ (*SSgg*)	27	0	27	1:0	--	--	17	10	27	1:1	0.178	1.815
	F_1 (*SsGg*)	Lil' Kim™ (*SSgg*)	37	0	37	1:0	--	--	18	19	37	1:1	0.869	0.027
$BC_{(P2)}$														
	F_1 (*SsGg*)	Blue Chiffon™ (*SsGG*)	90	37	127	3:1	0.282	1.157	87	3	90	1:0	--	--

[z]Generation (family) analyzed using the χ^2 goodness of fit test for expected segregation ratios. F_1 = cross between parent 1 and parent 2. F_2 = S_1 family resulting from self-pollination of F_1 family. $BC_{(P1)}$ = family created from the backcross of F_1 family to parent 1. $BC_{(P2)}$ = family created from the backcross of F_1 family to parent 2.

[y]Parent (including proposed genotype for *spotless* and *geisha*) used in reciprocal combinations with parent 2.

[x]Parent (including proposed genotype for *spotless* and *geisha*) used in reciprocal combinations with parent 1.

[w]Number of observed progeny with an exhibiting a red eyespot.

[v]Number of observed *spotless* progeny lacking an eyespot.

[u]Total number of progeny.

[t]Expected segregation ratio of eyespot to *spotless* progeny.

[s]Number of observed progeny with eyespot and colorful petal bodies.

[r]Number of observed progeny with *geisha* phenotype (eyespot plus white to blush pink petals).

[q]Total number of progeny (excluding *spotless* progeny).

[p]Expected segregation ratio of normal petals to *geisha* petals.

Table 8.5. Segregation of *spotless* and *geisha* phenotypes in F_1, F_2, and backcross generations from reciprocal crosses of *Hibiscus syriacus* Fiji™ and *H. syriacus* White Chiffon®.

Family[z]	Parent 1[y]	Parent 2[x]	Eyespot[w]	*Spotless*[v]	Total[u]	Segregation[t]	*P*	χ^2	Colorful[s]	*Geisha*[r]	Total[q]	Segregation[p]	*P*	χ^2
F_1														
	Fiji™ (*SSgg*)	White Chiffon® (*ssGg*)	61	0	61	1:0	--	--	25	36	61	1:1	0.159	1.984
F_2														
	F_1 (*Ssgg*)	F_1 (*Ssgg*)	7	1	8	3:1	0.414	0.667	0	6	6	0:1	--	--
	F_1 (*SsGg*)	F_1 (*SsGg*)	11	3	14	3:1	0.758	0.095	7	4	11	3:1	0.384	0.758
$BC_{(P2)}$														
	F_1 (*Ssgg*)	White Chiffon® (*ssGg*)	26	28	54	1:1	0.785	0.074	15	15	30	1:1	1.000	0.000
	F_1 (*SsGg*)	White Chiffon® (*ssGg*)	2	1	3	1:1	0.564	0.333	2	0	2	3:1	0.414	0.667

[z]Generation (family) analyzed using the χ^2 goodness of fit test for expected segregation ratios. F_1 = cross between parent 1 and parent 2. F_2 = S_1 family resulting from self-pollination of F_1 family. $BC_{(P2)}$ = family created from the backcross of F_1 family to parent 2.

[y]Parent (including proposed genotype for *spotless* and *geisha*) used in reciprocal combinations with parent 2.

[x]Parent (including proposed genotype for *spotless* and *geisha*) used in reciprocal combinations with parent 1.

[w]Number of observed progeny exhibiting a red eyespot.

[v]Number of observed *spotless* progeny lacking an eyespot.

[u]Total number of progeny.

[t]Expected segregation ratio of eyespot to *spotless* progeny.

[s]Number of observed progeny with eyespot and colorful petal bodies.

[r]Number of observed progeny with *geisha* phenotype (eyespot plus white to blush pink petals).

[q]Total number of progeny (excluding *spotless* progeny).

[p]Expected segregation ratio of normal petals to *geisha* petals.

Table 8.6. Segregation test for recessive epistasis of *spotless* over *geisha* in heterozygote (*SsGg*) self-pollinations of *Hibiscus syriacus*.

Family[z]	Parent 1	Parent 2	Colorful[y]	*Geisha*[x]	*Spotless*[w]	Total[v]	Segregation[u]	*P*	χ^2
S_1 (F_2)									
	'Woodbridge' (*SsGg*)	'Woodbridge' (*SsGg*)	35	12	19	66	9:3:4	0.774	0.512
S_1 (F_2)									
	F_1 family Lil' Kim™ x Blue Chiffon™ (*SsGg*)	F_1 family Lil' Kim™ x Blue Chiffon™ (*SsGg*)	55	10	14	79	9:3:4	0.056	5.748

[z]Generation(family) analyzed using the χ^2 goodness of fit test for expected segregation ratios. F_2 = S_1 family resulting from self-pollination of parents.

[y] Number of observed progeny with eyespot and colorful petal bodies.

[x]Number of observed progeny with eyespot and white to blush pink petals.

[w]Number of observed progeny with white petals and no eyespot.

[v]Total number of observed progeny

[u]Expected segregation ratio based on recessive epistasis of *spotless* over *geisha* phenotype.

Table 8.7. Segregation of blue flowers in in F_1, F_2, and backcrosses generations in *Hibiscus syriacus*.

Family[z]	Parent 1[y]	Parent 2[x]	Pink to lavender[w]	Blue [L* <65 a*<18.3 b* < -18.3][v]	Total[u]	Segregation[t]	*P*	χ^2
S_1	'Blue Bird'	'Blue Bird'	0	24	24	0:1	--	--
	Blue Chiffon™	Blue Chiffon™	0	2	2	0:1	--	--
	Blue Satin®	Blue Satin®	0	26	26	0:1	--	--
F_1								
	Blue Satin	'Blue Bird'	0	19	19	0:1	--	--
$F_{1\ (LK\|BC)}$								
	Lil' Kim™	Blue Chiffon™	28	0	28	1:0	--	--
F_2								
	$F_{1\ (LK\ \|\ BC)}$	$F_{1\ (LK\ \|\ BC)}$	92	1	93	???	--	--
$BC_{Blue\ Chiffon™}$								
	$F_{1\ (LK\ \|\ BC)}$	Blue Chiffon™	79	8	87	???	--	--

[z]Generation (family) analyzed using the $\chi 2$ goodness of fit test for expected segregation ratios. S_1 = family resulting from self-pollination. F_1 = family resulting from reciprocal cross pollination of parent 1 and parent 2. F_2 = S_1 family resulting from the self-pollination of the F_1 family created from the cross Lil' Kim™ x Blue Chiffon™. BC = backcross of the F_1 family (Lil' Kim™ x Blue Chiffon™) to Blue Chiffon™.

[y]Parent used in reciprocal combinations with parent 2.

[x]Parent used in reciprocal combinations with parent 1.

[w]Number of observed progeny with an eyespot and pink to lavender flowers.

[v]Number of observed progeny with an eyespot and blue flowers based on CIEL*a*b* estimates for true blue flowers.

[u]Total number of progeny (*spotless* phenotypes removed).

[t]Expected segregation ratio of pink/lavender flowers to blue flowers.

Table 8.8. Depth of color for each colorful flower (*spotless* and *geisha* phenotype removed) among cross combinations in *Hibiscus syriacus*.

Cross[z]	Depth of color (mean CIE L* ± SE)[y]	Average color (CIEL*a*b*)[x]	Nearest RHS value[w]	RHS group[v]
Blue Satin® x 'Pink Giant'	52.6 ± 1.3 A		77B	Purple
Blue Satin® x Blue Satin®	53.4 ± 1.0 A		90D	Violet-Blue
'Blue Bird' x 'Blue Bird'	55.5 ± 0.9 AB		90D	Violet-Blue
Blue Satin® | 'Minerva'	55.5 ± 1.4 A-C		N78C	Purple
'Minerva' x 'Blue Bird'	55.7 ± 1.1 A-C		N78C	Purple
'Blue Bird' | Blue Satin®	56.1 ± 1.0 BC		90D	Violet-Blue
'Aphrodite' x 'Aphrodite'	56.1 ± 1.0 BC		70B	Red-Purple
'Aphrodite' | 'Blue Bird'	56.4 ± 0.9 BC		N78C	Purple
'Aphrodite' | Blue Satin®	57.6 ± 0.9 B-D		N78C	Purple
'Blushing Bride' x 'Pink Giant'	57.9 ± 1.8 B-E		72D	Red-Purple
Lavender Chiffon™ | Strawberry Smoothie™	58.4 ± 0.8 C-E		N78C	Purple
'Woodbridge' x 'Minerva'	58.8 ± 1.6 C-E		N74C	Red-Purple
Pink Chiffon® x White Chiffon®	59.0 ± 0.7 DE		N78C	Purple
Fiji™ | Hawaii™	60.0 ± 0.7 D-F		N80C	Purple-Violet
Lavender Chiffon™ x White Chiffon®	60.1 ± 1.0 D-F		N81C	Purple-Violet
'Lucy' x 'Red Heart'	60.2 ± 1.4 D-F		N78C	Purple
Blue Satin® | 'Buddha Belly'	60.3 ± 0.3 EF		N81C	Purple-Violet
Blue Satin® | 'Woodbridge'	60.4 ± 0.9 EF		N78D	Purple
Blue Satin® | White Chiffon®	60.8 ± 0.6 EF		N80C	Purple-Violet
Blue Chiffon™ | Fiji™	61.0 ± 1.0 EF		N80C	Purple-Violet
'Aphrodite' x 'Minerva'	61.0 ± 1.0 E-G		N78D	Purple
Fiji™ | White Chiffon®	61.9 ± 1.0 F-H		N78D	Purple
Blue Satin® | 'Red Heart'	62.0 ± 1.0 F-H		N80C	Purple-Violet
'Aphrodite' | Bali™	62.2 ± 0.5 F-H		N80C	Purple-Violet
'Blue Bird' | China Chiffon™	62.2 ± 0.8 F-H		N80C	Purple-Violet
'Blushing Bride' x Blue Chiffon™	62.5 ± 0.8 F-H		N80C	Purple-Violet
'Aphrodite' | 'Woodbridge'	62.7 ± 0.9 F-I		N78D	Purple
Bali™ | 'Blue Bird'	63.3 ± 0.6 G-I		N80C	Purple-Violet
China Chiffon™ x Blue Chiffon™	64.1 ± 0.9 H-J		N80C	Purple-Violet
'Aphrodite' | 'Red Heart'	64.9 ± 0.7 I-K		N80C	Purple-Violet
'Woodbridge' x 'Woodbridge'	64.9 ± 0.9 I-K		75A	Purple
'Blue Bird' | Lil' Kim™	65.1 ± 0.5 I-K		N80C	Purple-Violet

Table 8.8 (continued). Depth of color for each colorful flower (*spotless* and *geisha* phenotype removed) among cross combinations in *Hibiscus syriacus*.

Cross[z]	Color depth (mean CIE L* ± SE)[y]	Average color (CIEL*a*b*)[x]	Nearest RHS value[w]	RHS group[v]
Lil' Kim™ | White Chiffon®	66.0 ± 0.6 J-L		N80C	Purple-Violet
Blue Satin® | 'Diana'	66.3 ± 0.4 K-M		N81D	Purple-Violet
'Aphrodite' | 'Diana'	66.8 ± 0.7 K-N		84B	Violet
Lil' Kim™ | 'Woodbridge'	67.3 ± 0.9 K-N		84B	Violet
Lil' Kim™ x Blue Chiffon™	67.8 ± 0.5 L-N		N80D	Purple-Violet
Blue Chiffon™ | 'Red Heart'	67.9 ± 0.4 MN		N81D	Purple-Violet
Blue Chiffon™ | 'Diana'	68.3 ± 0.4 N		N81D	Purple-Violet

[z]Unidirectional crosses represented: female x male. Reciprocal crosses represented: Parent 1 | Parent 2.

[y]Average pigment production of progeny exhibiting colorful flowers (*spotless* and *geisha* phenotypes removed). CIE L* value used to represent total pigment production irrespective of hue. Lower L* value represents higher pigment production. Averages were calculated for crosses with at least ten progeny. Means sharing letters are not significantly different.

[x]Average color of colorful flowers for each cross measured with a colorimeter.

[w]Nearest color value in the Royal Horticulture Society (RHS) Colour Chart.

[v]Color group in the RHS Colour Chart.

Table 8.9. Petal number variation among flower forms and individual crosses in *Hibiscus syriacus*.

Flower form cross	Cross	Petal no. (mean ± SE)
Double | Double		36.0 ± 2.4 A
	Pink Chiffon® x Pink Chiffon®	52.9 ± 1.6 A
	White Chiffon® x Strawberry Smoothie™	51.3 ± 3.0 A
	Lavender Chiffon™ | Strawberry Smoothie™	43.3 ± 2.2 B
	Blue Chiffon™ x White Chiffon®	38.1 ± 2.4 C
	Blue Chiffon™ | Fiji™	37.6 ± 1.8 C
	Pink Chiffon® x White Chiffon®	35.5 ± 1.4 C
	White Chiffon® x White Chiffon®	33.4 ± 1.3 CD
	Lavender Chiffon™ x Pink Chiffon®	32.7 ± 2.8 C-E
	Lavender Chiffon™ x White Chiffon®	31.2 ± 1.9 DE
	'Blushing Bride' x Fiji™	31.0 ± 4.4 DE
	'Blushing Bride' x Blue Chiffon™	29.5 ± 2.7 EF
	China Chiffon™ x Blue Chiffon™	25.9 ± 1.5 FG
	Fiji™ | White Chiffon®	25.2 ± 1.4 G
Single | Double		13.9 ± 1.0 B
	Blue Chiffon™ | 'Red Heart'	21.1 ± 0.9 H
	Blue Chiffon™ | 'Diana'	18.9 ± 0.9 HI
	'Buddha Belly' x White Chiffon®	18.8 ± 1.9 HI
	'Diana' | White Chiffon®	18.4 ± 0.9 I
	'Aphrodite' x Fiji™	16.8 ± 2.7 IJ
	'Blushing Bride' x 'Pink Giant'	15.5 ± 3.8 I-K
	'Aphrodite' | Bali™	14.9 ± 0.8 JK
	Bali™ | 'Diana'	14.1 ± 0.8 J-L
	Fiji™ x Hawaii™	13.2 ± 1.4 J-M
	'Diana' x Fiji™	12.5 ± 2.3 J-N
	Blue Satin® | White Chiffon®	12.2 ± 0.4 K-N
	'Lucy' x 'Red Heart'	10.4 ± 2.2 L-O
	'Blue Bird' | China Chiffon™	9.9 ± 0.5 M-O
	Bali™ | 'Blue Bird'	9.8 ± 0.4 M-O
	Lil' Kim™ | White Chiffon®	8.7 ± 0.5 N-Q
	Lil' Kim™ x Blue Chiffon™	6.8 ± 0.4 O-Q
Single | Single		5.9 ± 0.2 C
	'Diana' | 'Red Heart'	9.7 ± 0.4 M-O
	'Aphrodite' x 'Aphrodite'	9.4 ± 1.3 M-P
	'Red Heart' x 'Red Heart'	8.9 ± 1.2 M-Q

Table 8.9 (continued). Petal number variation among flower forms and individual crosses in *Hibiscus syriacus*.

Flower form cross	Cross	Petal no. (mean ± SE)
Single | Single (continued)		
	'Aphrodite' | 'Diana'	6.5 ± 0.3 O-Q
	'Woodbridge' x 'Pink Giant'	6.2 ± 1.1 O-Q
	'Buddha Belly' | 'Diana'	6.1 ± 0.2 O-Q
	Blue Satin® x 'Pink Giant'	6.0 ± 0.7 O-Q
	'Aphrodite' | 'Red Heart'	6.0 ± 0.3 O-Q
	Blue Satin® | 'Minerva'	5.8 ± 0.3 O-Q
	'Aphrodite' x 'Minerva'	5.7 ± 0.4 O-Q
	'Diana' x 'Minerva'	5.6 ± 0.4 O-Q
	Blue Satin® | 'Diana'	5.5 ± 0.1 O-Q
	Blue Satin® | 'Buddha Belly'	5.5 ± 0.1 O-Q
	Blue Satin® | 'Red Heart'	5.5 ± 0.2 O-Q
	'Aphrodite' | Blue Satin®	5.4 ± 0.2 O-Q
	'Diana' | 'Woodbridge'	5.4 ± 0.2 O-Q
	'Minerva' x 'Blue Bird'	5.3 ± 0.1 O-Q
	Blue Satin® | 'Woodbridge'	5.3 ± 0.2 PQ
	'Blue Bird' | Blue Satin®	5.3 ± 0.1 PQ
	'Aphrodite' | 'Blue Bird'	5.3± 0.1 PQ
	Blue Satin® x Blue Satin®	5.3 ± 0.1 PQ
	'Blue Bird' x 'Blue Bird'	5.2 ± 0.1 PQ
	'Aphrodite' | 'Woodbridge'	5.2 ± 0.1 PQ
	'Woodbridge' x 'Minerva'	5.2 ± 0.1 PQ
	'Blue Bird' | Lil' Kim™	5.2 ± 0.1 PQ
	'Diana' x Lil' Kim™	5.1 ± 0.1 PQ
	'Woodbridge' x 'Woodbridge'	5.1 ± 0.0 Q
	'Red Heart' | 'Woodbridge'	5.1 ± 0.1 Q
	Lil' Kim™ | 'Woodbridge'	5.1 ± 0.0 Q

[z]Reciprocal (" | ") cross combinations among flower types including single-flowered forms with five petals and double-flowered forms with numerous petaloid stamen.

[y]Individual crosses with unidirectional crosses represented: female x male and reciprocal crosses represented: Parent 1 | Parent 2.

[x]Petal area as an estimate of flower size measured as length x width.

Table 8.10. Petal size variation among flower forms and individual crosses in *Hibiscus syriacus*.

Flower form cross[z]	Cross[y]	Petal area (mean mm^2 ± SE)[x]
Single | Double		2998 ± 188 A
	'Blue Bird' | China Chiffon™	4314 ± 122 A
	Blue Chiffon™ | 'Diana'	4101 ± 85 A
	'Diana' x Fiji™	3910 ± 251 AB
	'Aphrodite' | Bali™	3728 ± 90 B
	Blue Chiffon™ | 'Red Heart'	3400 ± 113 C
	Bali™ | 'Blue Bird'	3129 ± 112 D
	Bali™ | 'Diana'	3049 ± 127 DE
	'Diana' | White Chiffon®	3034 ± 79 DE
	Lil' Kim™ x Blue Chiffon™	2988 ± 124 D-F
	Blue Satin® | White Chiffon®	2949 ± 76 D-F
	Fiji™ | Hawaii™	2560 ± 91 F-J
	Lil' Kim™ | White Chiffon®	2468 ± 78 G-K
	'Buddha Belly' x White Chiffon®	2317 ± 101 I-L
	'Aphrodite' x Fiji™	2242 ± 148 I-M
	'Blushing Bride' x 'Pink Giant'	1971 ± 170 K-O
	'Lucy' x 'Red Heart'	1816 ± 128 M-O
Single | Single		2379 ± 101 B
	'Red Heart' x 'Red Heart'	3646 ± 365 BC
	'Diana' | 'Red Heart'	3619 ± 86 BC
	'Aphrodite' | 'Diana'	3549 ± 138 BC
	'Aphrodite' | 'Red Heart'	3050 ± 160 DE
	Blue Satin® | 'Diana'	2996 ± 121 D-F
	'Aphrodite' x 'Aphrodite'	2800 ± 111 E-H
	Blue Satin® | 'Buddha Belly'	2555 ± 46 F-J
	'Buddha Belly' | 'Diana'	2482 ± 72 G-K
	'Blue Bird' x 'Blue Bird'	2461 ± 131 G-K
	'Minerva' x 'Blue Bird'	2447 ± 106 G-L
	Blue Satin® | 'Minerva'	2443 ± 131 H-L
	'Aphrodite' | 'Blue Bird'	2436 ± 89 I-L
	'Blue Bird' | Lil' Kim™	2372 ± 104 I-L
	Blue Satin® | 'Red Heart'	2270 ± 130 I-M
	'Aphrodite' | Blue Satin®	2223 ± 82 I-M
	'Aphrodite' x 'Minerva'	2203 ± 95 I-N

Table 8.10 (continued). Petal size variation among flower forms and individual crosses in *Hibiscus syriacus*.

Flower form cross[z]	Cross[y]	Petal area (mean mm^2 ± SE)[x]
Single | Single (continued)		
	'Diana' x Lil' Kim™	2187 ± 105 I-N
	Blue Satin® x 'Pink Giant'	2122 ± 105 J-N
	'Aphrodite' | 'Woodbridge'	2095 ± 106 J-N
	Blue Satin® | 'Woodbridge'	1975 ± 85 K-N
	'Woodbridge' x 'Woodbridge'	1973 ± 63 K-N
	Blue Satin® x Blue Satin®	1969 ± 64 L-O
	Lil' Kim™ | 'Woodbridge'	1964 ± 126 L-O
	'Diana' | 'Woodbridge'	1947 ± 86 L-O
	'Red Heart' | 'Woodbridge'	1930 ± 105 L-O
	'Woodbridge' x 'Minerva'	1929 ± 136 L-O
	'Blue Bird' | Blue Satin®	1890 ± 86 L-O
	'Diana' x 'Minerva'	1847 ± 102 L-O
	'Woodbridge' x 'Pink Giant'	1611 ± 94 NO
Double | Double		2309 ± 124 B
	Lavender Chiffon™ | Strawberry Smoothie™	2903 ± 166 D-G
	China Chiffon™ x Blue Chiffon™	2900 ± 96 D-G
	Blue Chiffon™ x White Chiffon®	2671 ± 165 E-I
	Fiji™ | White Chiffon®	2658 ± 87 F-I
	Lavender Chiffon™ x White Chiffon®	2593 ± 109 F-I
	Lavender Chiffon™ x Pink Chiffon®	2538 ± 155 F-K
	White Chiffon® x Strawberry Smoothie™	2272 ± 182 I-M
	Pink Chiffon® x Pink Chiffon®	2209 ± 76 I-M
	Blue Chiffon™ | Fiji™	2155 ± 76 J-N
	'Blushing Bride' x Blue Chiffon™	1953 ± 140 L-O
	White Chiffon® x White Chiffon®	1849 ± 60 L-O
	Pink Chiffon® x White Chiffon®	1846 ± 61 M-O
	'Blushing Bride' x Fiji™	1470 ± 160 O

[z]Reciprocal (" | ") cross combinations among flower types including single-flowered forms with five petals and double-flowered forms with numerous petaloid stamens.

[y]Individual crosses with unidirectional crosses represented: female x male and reciprocal crosses represented: Parent 1 | Parent 2.

[x]Petal area as an estimate of flower size measured as length x width.

Table 8.11. Flower phenotypes (eyespot, petal area, and color) and genotypes (*spotless* and *geisha*) for cultivars of *Hibiscus syriacus*.

Cultivar	eyespot	avg petal area (mm)	avg petal no.	avg CIE L*	avg CIE a*	avg CIE b*	CIEL*a*b* color	Nearest RHS no.	RHS Fan no.	RHS Group	*spotless* genotype	*geisha* genotype
Aphrodite	yes	1919	5	60.6	37.1	-12.5		72D	2	Red-Purple	*SS*	*Gg*
Bali™	yes	1292	17	90.5	0.3	1.2		N155B	4	White	*SS*	*gg*
Blue Bird	yes	1963	5	64.2	9.7	-18.1		85A	2	Violet	*Ss*	*GG*
Blue Chiffon™	yes	2511	38	55.9	11.3	-22.7		90D	2	Violet-Blue	*Ss*	*GG*
Blue Satin®	yes	1603	5	51.2	12.5	-24.6	mv	90D	2	Violet-Blue	*Ss*	*GG*
Blushing Bride	yes	659	45	89.5	2.1	5.1		N155C	4	White	*SS*	*gg*
Buddha Belly	no	mv	5	92.8	0.3	3.0		N155D	4	White	*ss*	*GG*
China Chiffon™	yes	2233	30	89.2	-0.7	3.8		N155D	4	White	*SS*	na
Diana	no	1902	5	90.8	-1.6	3.4		N155D	4	White	*ss*	*gg*
Fiji™	yes	1214	14	85.3	10.6	0.2		65D	2	Red-Purple	*SS*	*gg*
Hawaii™	yes	mv	mv	mv	mv	mv	mv	mv	mv	mv	mv	*GG*
Helene	yes	mv	5	mv	mv	mv	mv	mv	mv	mv	mv	*gg*
Lavender Chiffon™	yes	2258	17	71.9	21.9	-13.7		77D	2	Purple	*SS*	*Gg*
Lil Kim	yes	684	5	89.4	2.1	2.1		N155B	4	White	*SS*	*gg*
Lucy	yes	881	34	53.9	43.0	-10.5		70B	2	Red-Purple	*SS*	*GG*
Minerva	yes	2541	5	67.0	28.9	-16.0		75A	2	Purple	*Ss*	*Gg*
Pink Chiffon®	yes	mv	mv	mv	mv	mv	mv	mv	mv	mv	*SS*	*gg*
Pink Giant	yes	2113	5	50.5	41.3	-11.1		70B	2	Red-Purple	na	na
Raspberry Smoothie™	yes	mv	mv	46.8	49.8	-17.0		71C	2	Red-Purple	na	na
Red Heart	yes	2729	9	90.9	-1.1	3.0		N155D	4	White	*Ss*	*gg*
Strawberry Smoothie™	yes	mv	mv	88.9	9.1	1.1		56C	1	Red	*SS*	*gg*
White Chiffon®	no	1517	32	92.4	-0.6	6.6		N155D	4	White	*ss*	*Gg*
Woodbridge	yes	1858	5	74.2	21.5	-13.9		77D	2	Purple	*Ss*	*Gg*

Figures

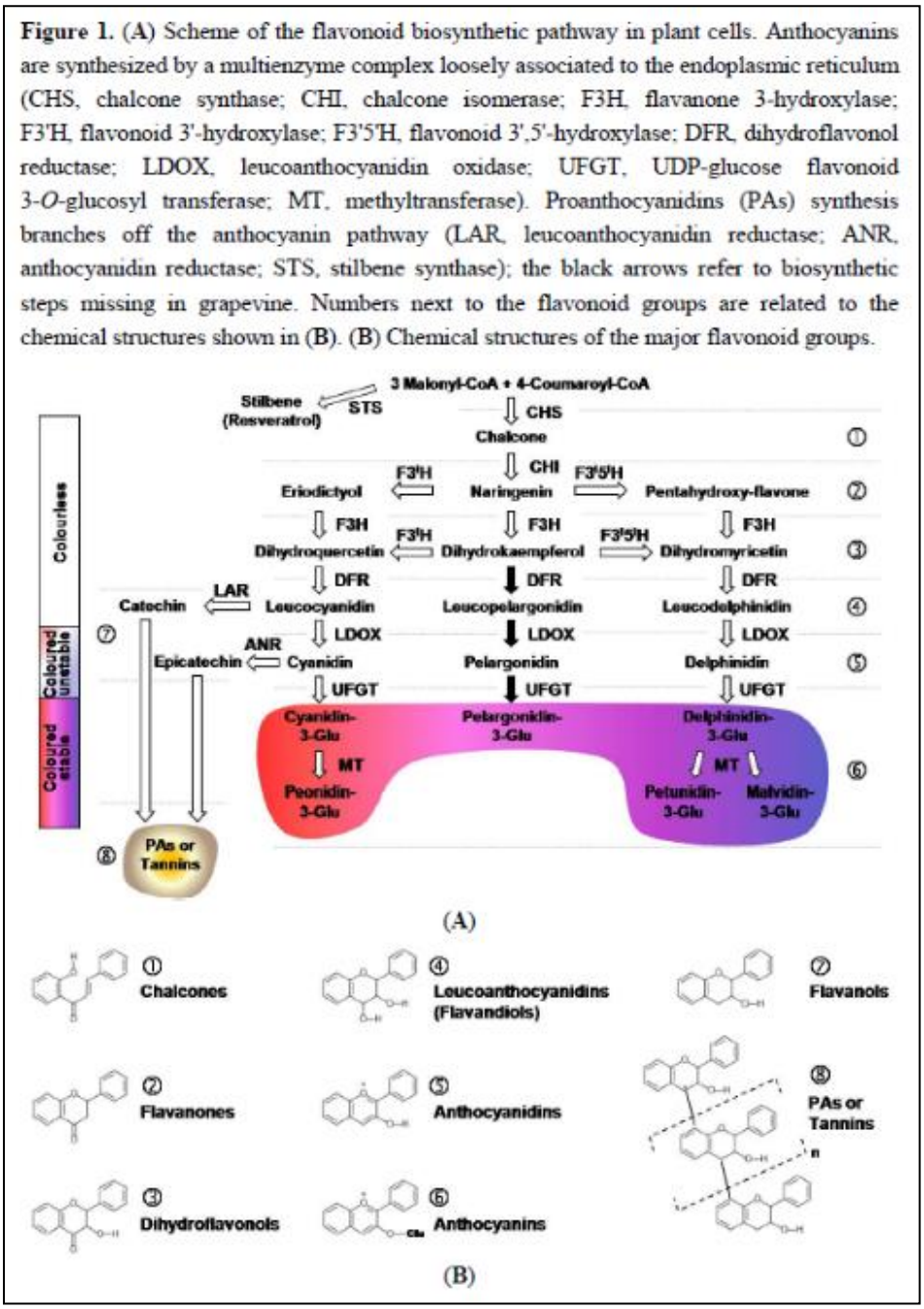

Fig. 8.1. Anthocyanins end products of the flavonoid biosynthetic pathway in plant cells, from Petrussa et al. (2013). Eyespots of *H. syriacus* composed mostly of cyanidins while the main petal body is composed of variable levels and combinations of six anthocyanins (Kim et al., 1989).

Fig. 8.2. Pollinations of *Hibiscus syriacus.* (A) Pollinations were performed in summer in a glasshouse kept free of pollinators. Each cross was labelled with a jeweler's tag and flowers were monitored for capsule development. (B) Capsule development post-pollination; capsules were monitored and collected at dehiscence as sutures began to open.

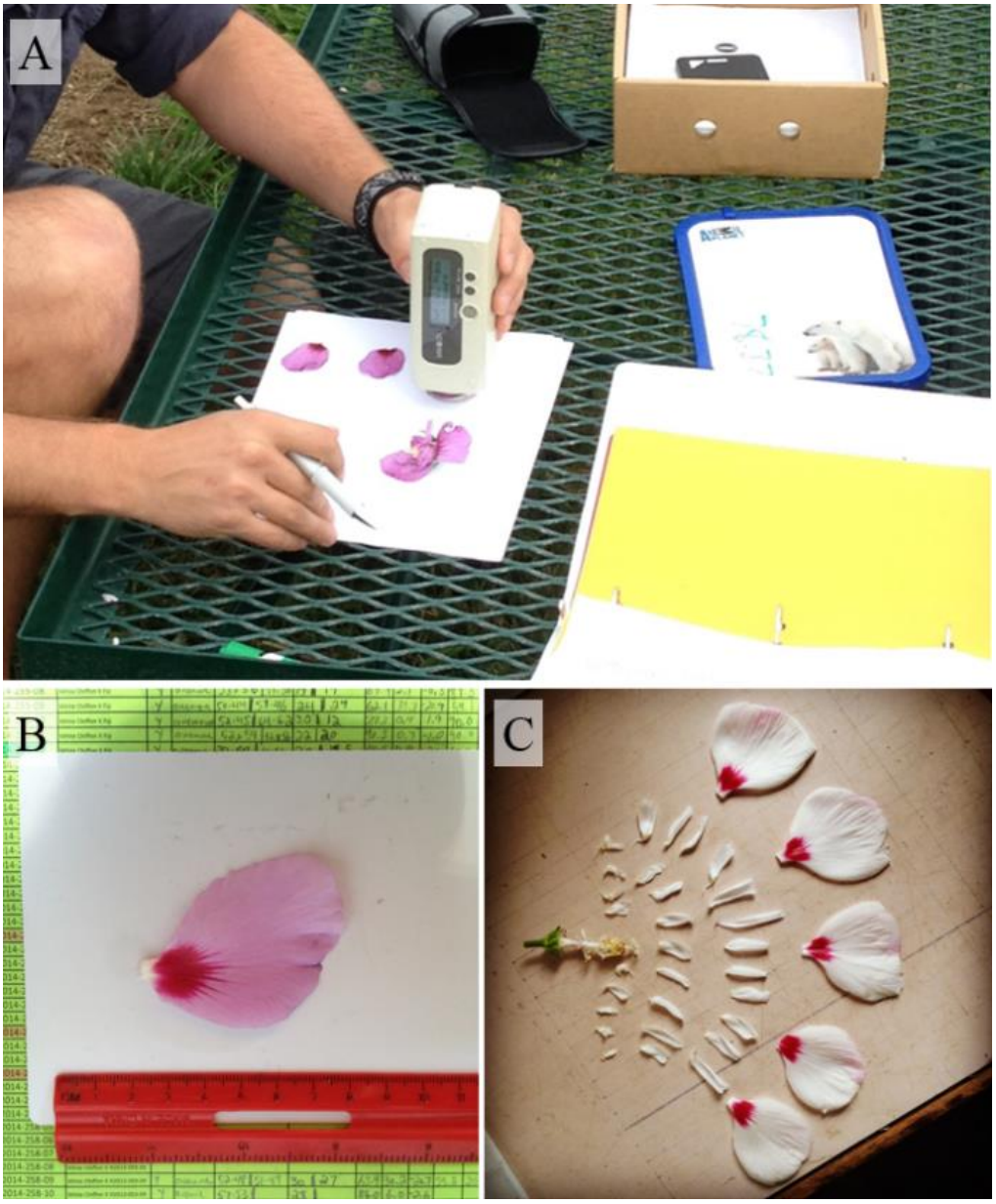

Fig. 8.3. Measurement of floral traits in hybrid progeny of *Hibiscus syriacus* L. (A) Colorimetric measurements of petals to generate CIE L*a*b* values. (B) Measurement of petal area (length × width). (C) Measurement of petal number including true petals and petaloid stamens.

A	B	C	D	E	F	G	H	I	J	K	L	M	N
Click for Instructions			RHS L*	RHS a*	RHS b*	RHS Fan	RHS Group	RHS Value	ΔE	Calculate		Clear Contents	
L*	a*	b*											
65.1	24	-19.3	63.7	24.5	-19.5	Fan 2	Purple-Violet Group	N80C	1.5	Hide or display RHS L*a*b*			
92	-1.15	1.85	92.1	2.2	1.5	Fan 4	White Group	N155B	3.36972	Hide or display color difference			
64	25.5	-21	63.7	24.5	-19.5	Fan 2	Purple-Violet Group	N80C	1.82757				
64.75	21.75	-21.15	63.7	24.5	-19.5	Fan 2	Purple-Violet Group	N80C	3.37454				
91.6	-0.9	0.1	92.1	2.2	1.5	Fan 4	White Group	N155B	3.43802				
91.25	-1.5	3.45	92.6	1.2	5.2	Fan 4	White Group	N155D	3.48927				
72.5	15.7	-15.55	70.1	20.4	-16.9	Fan 2	Purple Group	76A	5.44725	Save Results to New Tab			
54.2	32.6	-23.2	52.7	31.3	-21.2	Fan 2	Purple Group	77B	2.8178				
91.5	-1.6	1.4	92.1	2.2	1.5	Fan 4	White Group	N155B	3.84838				
91.2	-1.1	3.4	92.6	1.2	5.2	Fan 4	White Group	N155D	3.23883				
90.3	-1.25	3.4	92.6	1.2	5.2	Fan 4	White Group	N155D	3.81215				
62.8	22.65	-20.55	63.7	24.5	-19.5	Fan 2	Purple-Violet Group	N80C	2.30976				
66.6	19.7	-18.45	67.2	20.5	-17.4	Fan 2	Purple-Violet Group	N81D	1.45				
91	-1.5	1.7	92.1	2.2	1.5	Fan 4	White Group	N155B	3.86523				
54.35	28.5	-24.5	57.7	28.3	-25.1	Fan 2	Purple-Violet Group	N81C	3.40918				
90.85	-0.9	2.4	92.1	2.2	1.5	Fan 4	White Group	N155B	3.46157				
58.1	32.95	-20.25	56.8	33.7	-21.4	Fan 2	Purple Group	N78C	1.89077				
61.15	31.5	-20.3	56.8	33.7	-21.4	Fan 2	Purple Group	N78C	4.99725				
90.45	-0.95	1.4	92.1	2.2	1.5	Fan 4	White Group	N155B	3.55739				

Fig 8.4. Colorimetry macro utility for reporting CIE L*a*b* values in several hybrids of *Hibiscus syriacus* Blue Satin® x White Chiffon®. Program calculates the color difference (ΔE) between each sample and all 884 colors in the RHS Colour Chart. For each sample, it reports the color difference, the closest value in the RHS Colour Chart, and a colored cell corresponding to the RHS color.

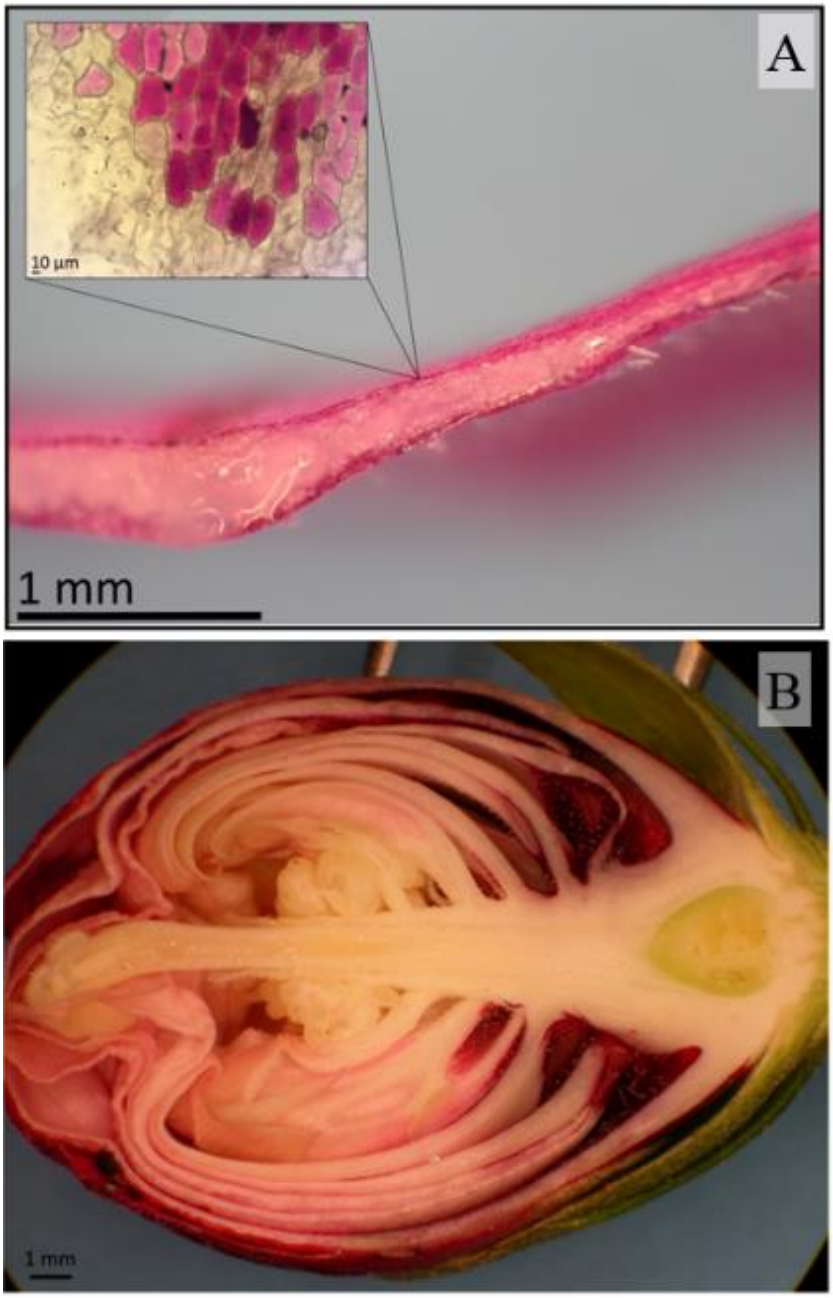

Fig. 8.5. Cross-section of petal tissue in *Hibiscus syriacus* ‘Blushing Bride’. (A) Cross-section of petal revealing the pigment production isolated to the cuticle layer. Inset: Cuticle peel near the junction between the petal body and the eyespot showing variable accumulation of anthocyanins. (B) Longitudinal section of a receptive flower bud showing true petals, petaloid stamens, and a functional pistil.

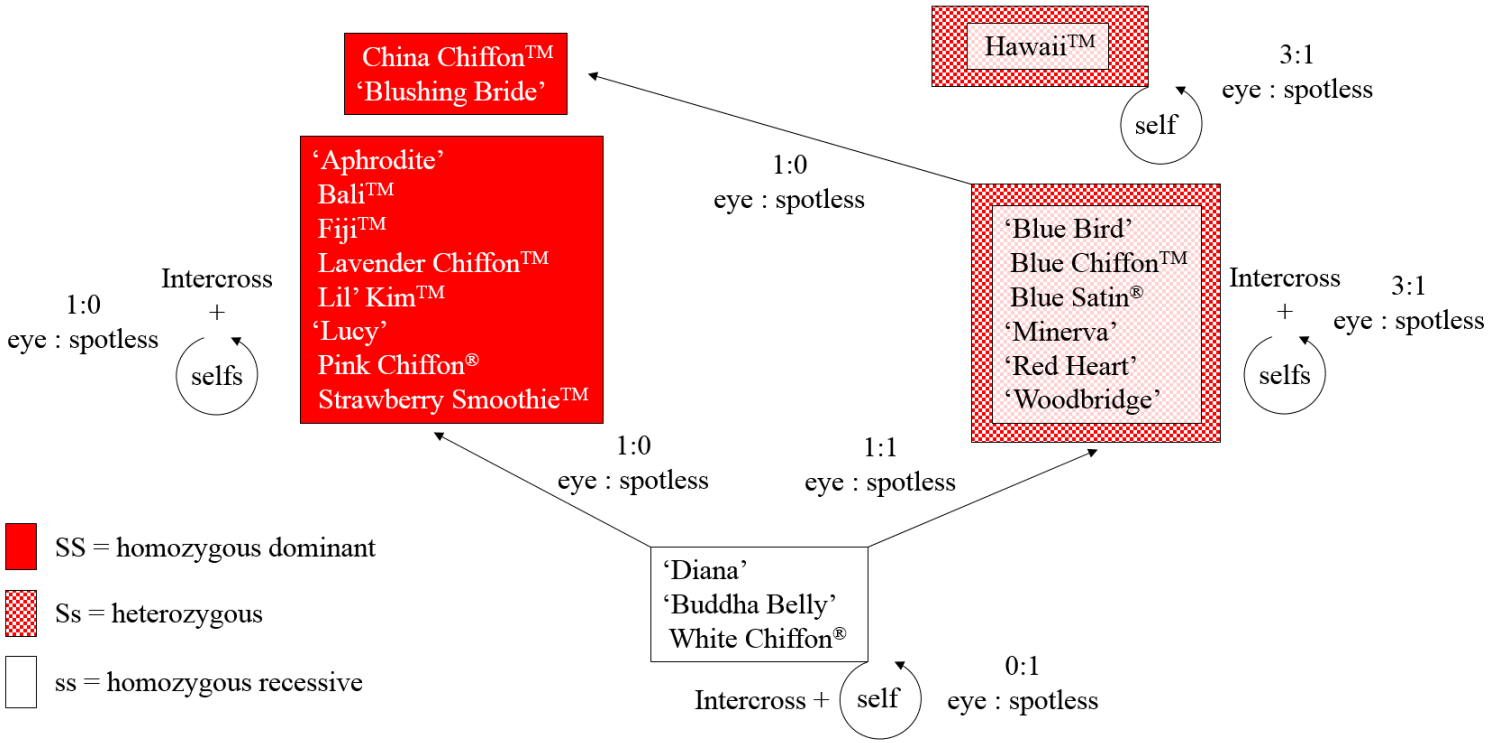

Fig. 8.6. Testcrosses used to predict *Hibiscus syriacus* genotypes for the recessive *spotless* gene. Self-pollinations of White Chiffon® and reciprocal crosses among 'Diana', 'Buddha Belly', and White Chiffon® resulted in only *spotless* phenotypes, confirming this group as homozygous recessive. Reciprocal cross combinations between the *spotless* taxa ('Diana', 'Buddha Belly', and White Chiffon®) and a diverse set of taxa exhibiting eyespots revealed two groups. The putative homozygous dominant group produced a 1:0 ratio of eyespot : *spotless* progeny in these testcrosses, as well as self-pollinations and cross-pollinations within the group. The putative heterozygous group produced a 1:1 ratio of eyespot : *spotless* progeny when crossed with the homozygous recessive group. Self-pollinations and cross-pollinations within the putative heterozygous group revealed 3:1 ratio of eyespot : *spotless* progeny. Taxa from the heterozygous group were then used to confirm homozygous dominant genotypes of two taxa (China Chiffon™ and 'Blushing Bride') that were not crossed with the recessive group. Further, self-pollinations of another taxa (Hawaii™) were used to confirm its genotype as heterozygous.

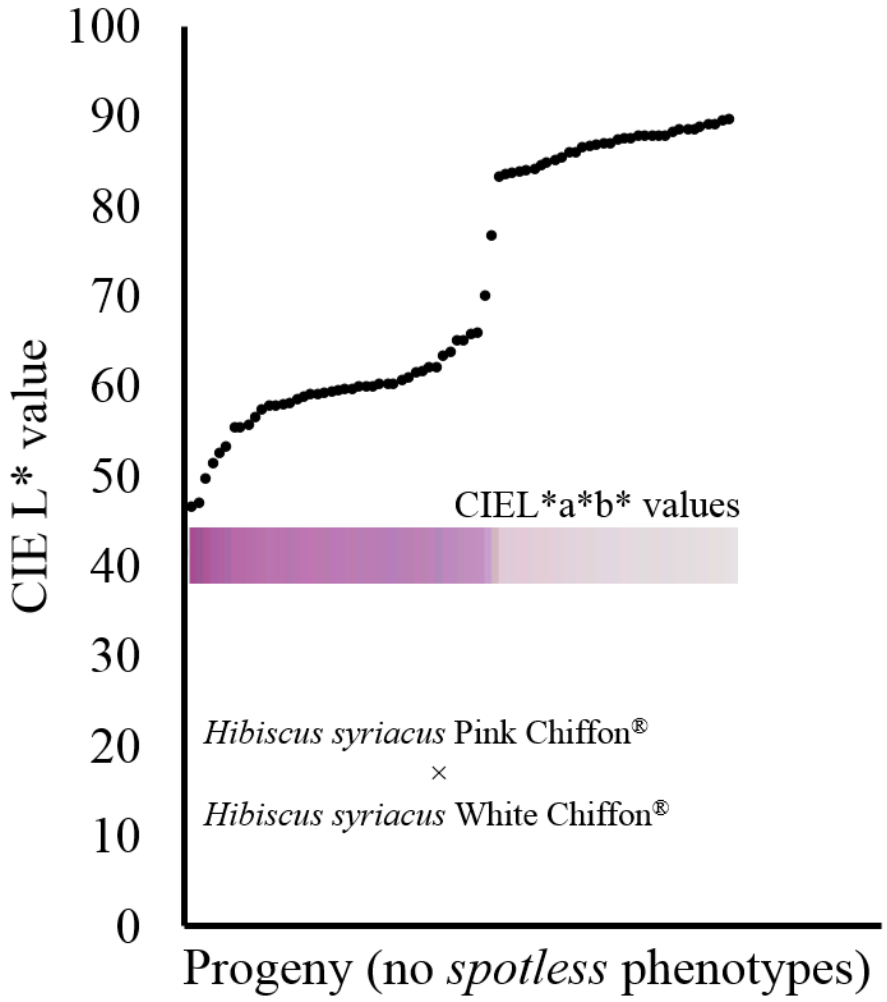

Fig. 8.7. *Hibiscus syriacus* progeny from the cross Pink Chiffon® × White Chiffon® segregating for dark pink progeny and blush to white progeny. No *spotless* phenotypes recovered. Progeny sorted from lowest to highest CIE L* value. Color bar represents the flower color each individual seedling based on recorded CIEL*a*b* values using a colorimeter.

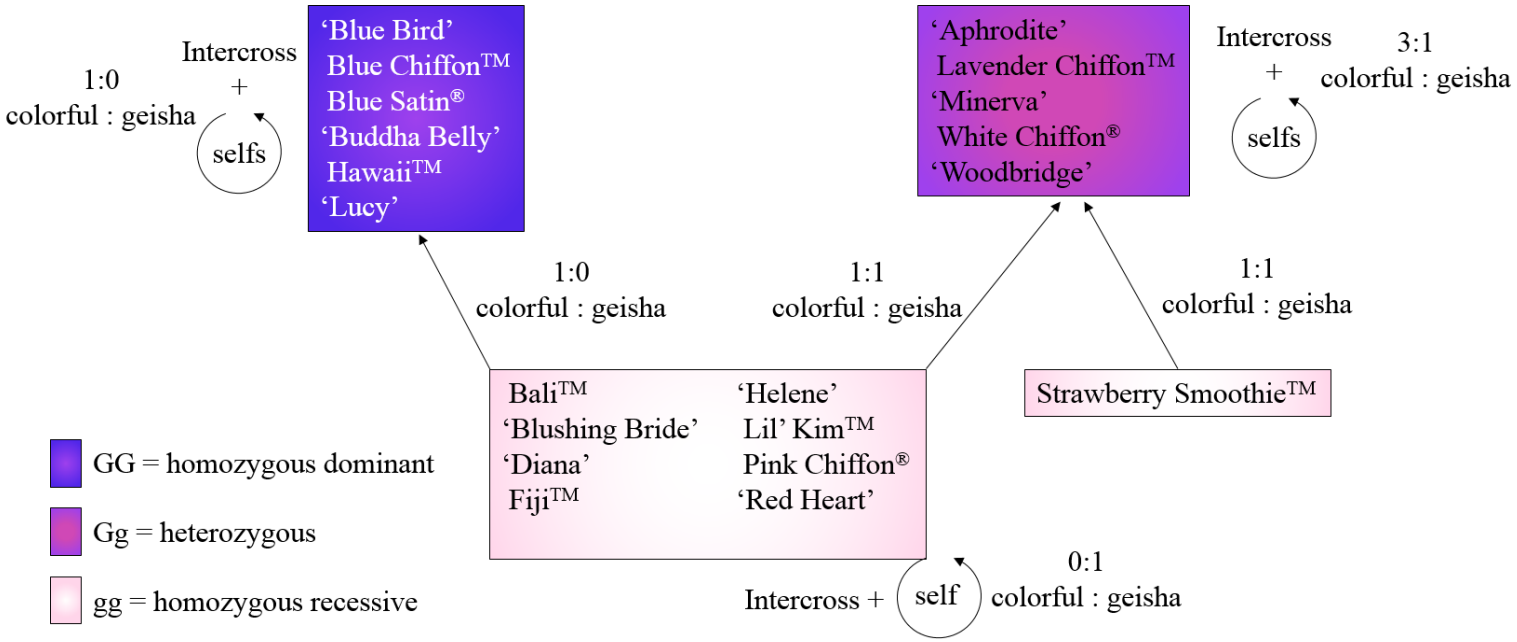

Fig. 8.8. Testcrosses used to predict *Hibiscus syriacus* genotypes for the recessive *geisha* gene. Self-pollinations and reciprocal crosses among Bali™, 'Blushing Bride', 'Diana', Fiji™, 'Helene', Lil' Kim™, Pink Chiffon®, and 'Red Heart' resulted in only *geisha* phenotypes, confirming this group as homozygous recessive. Reciprocal crosses were performed between *geisha* taxa and a diverse set of taxa exhibiting full color production in the petal body. Two groups were revealed based on their segregation ratios. Taxa exhibiting segregation ratios of 1:0 (colorful : *geisha*) were classified as homozygous dominant. Taxa exhibiting segregation ratios of 1:1 (colorful : *geisha*) were classified as heterozygous. One taxon, Strawberry Smoothie™, not included in recessive self-pollinations or intercrosses was added to the homozygous recessive group based on its 1:1 segregation ratios (colorful : *geisha*) with the heterozygous group. Self-pollinations and intercrosses within the homozygous dominant group and within the heterozygous group further confirmed their genotypes based on segregation of the *geisha* phenotype.

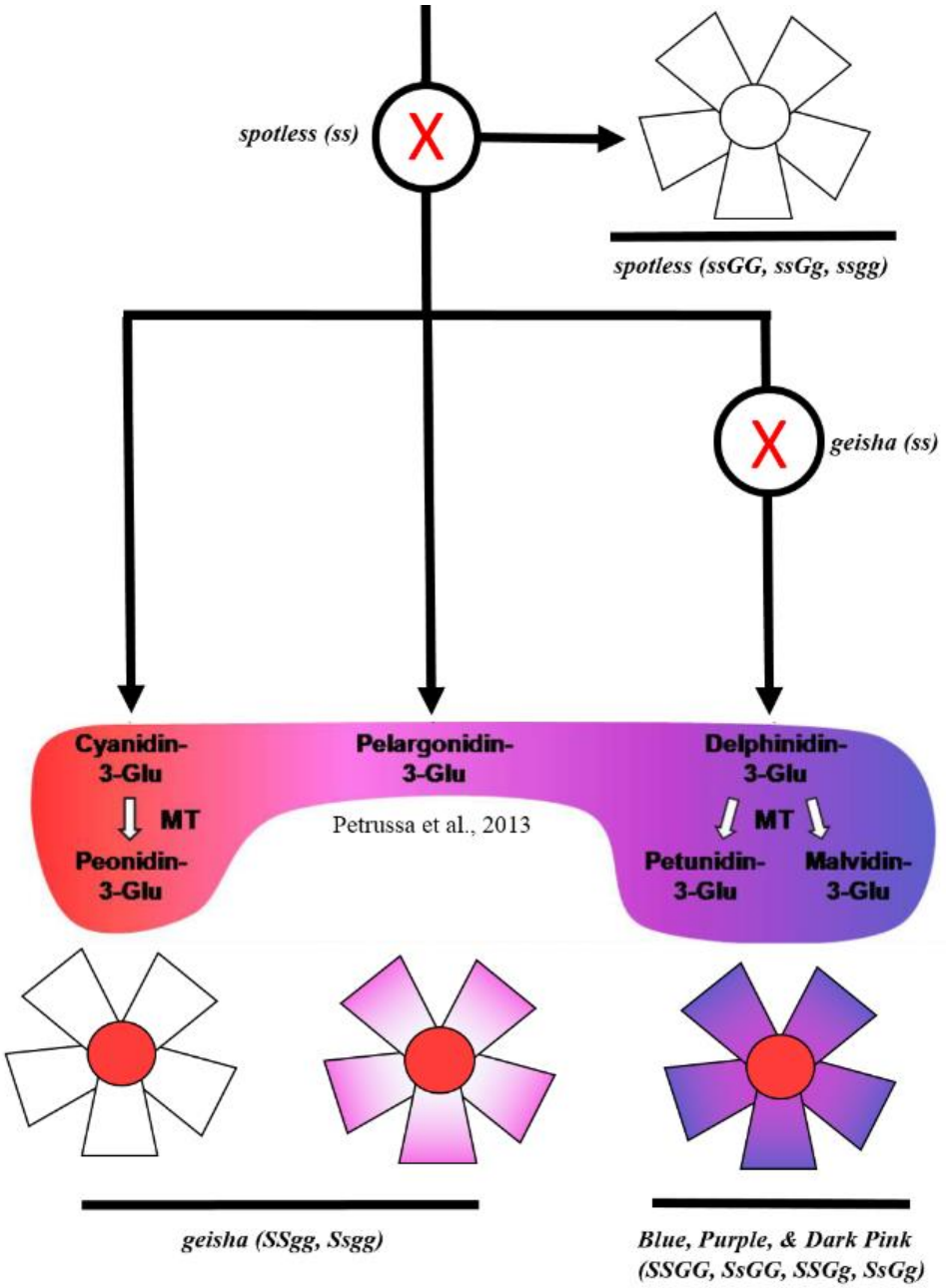

Fig. 8.9. Proposed *Hibiscus syriacus* flower phenotypes and genotypes, and proposed gene pathway arranged on a simplified flavonoid biosynthetic pathway from Petrussa et al., 2013. Flowers with no pigment production controlled by a recessive gene upstream in the flavonoid biosynthetic pathway called *spotless*, resulting in pure, white flowers. Flowers with at least one dominant allele for *spotless* result in flowers with eyespots. Of the flowers with eyespots, color segregation is controlled by another recessive mutation called *geisha* that disrupts the delphinidin biosynthetic pathway. Flowers homozygous recessive for *geisha* result in cyanidin, peonidin, and pelargonidin type flowers. Since red is not expressed in the petal body, cyanidin rich flowers express as white with an eyespot. Flowers with more peonidin and pelargonidin pigments express as blush pink.

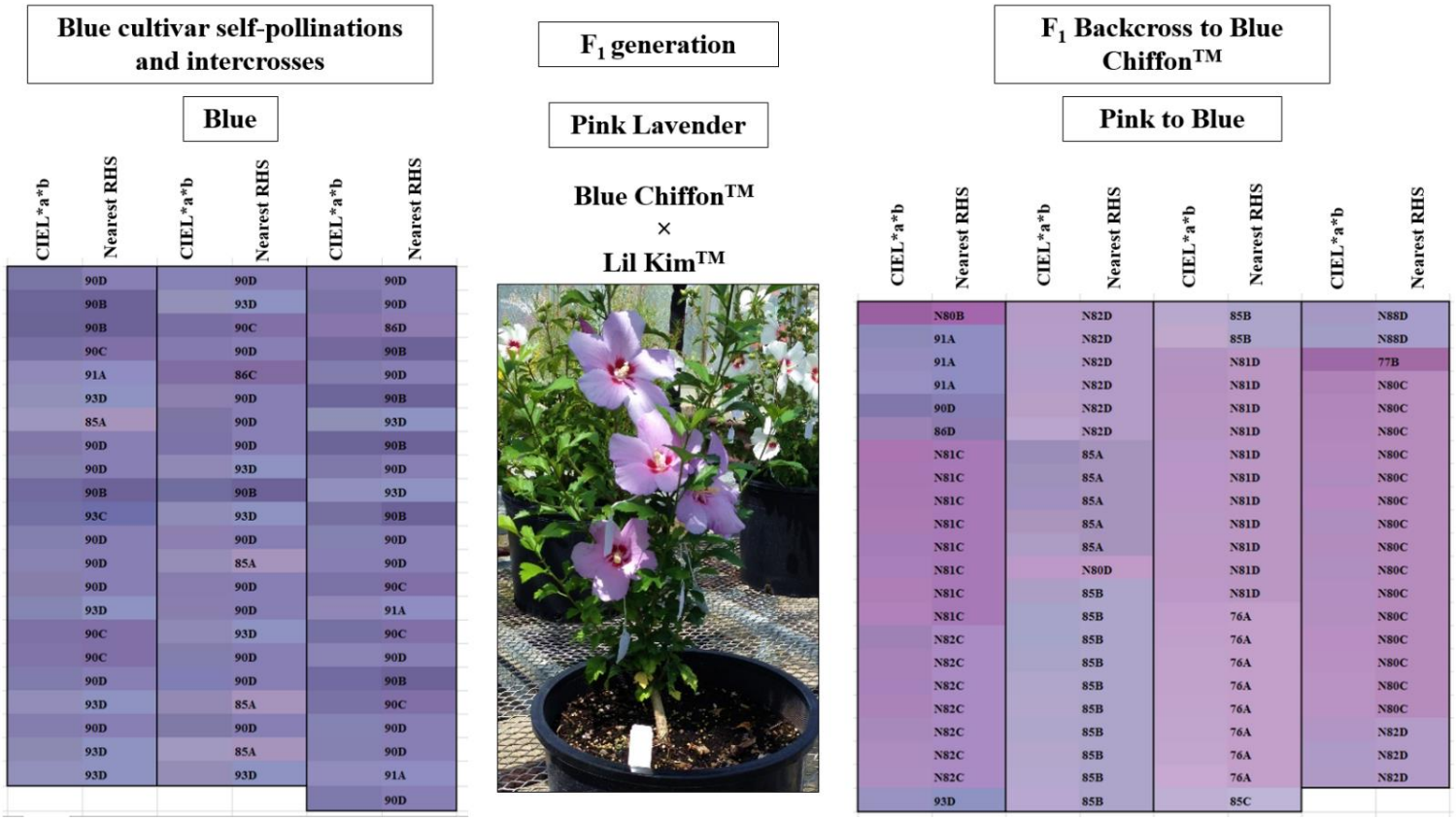

Fig. 8.10. Blue color segregation in *Hibiscus syriacus*. Colored cells indicate the CIEL*a*b* value for each seedling, and adjacent cells represents the nearest RHS value based on the color difference equation. Left: true blue flower color recovered from self-pollinations and intercrosses among blue taxa ('Blue Bird', Blue Chiffon™, Blue Satin®). Center: F_1 hybrids with all other cultivars tested yielded pink to lavender flowers. Right: F_1 backcrosses to Blue Chiffon™ yielded few true blue flowers (CIE L*< 65, a*< 18.3, b* < -18.3).

Fig. 8.11. Flower of *Hibiscus syriacus* with improved depth of color (low CIE L*) were recovered from the cross Blue Satin® x ‘Pink Giant’.

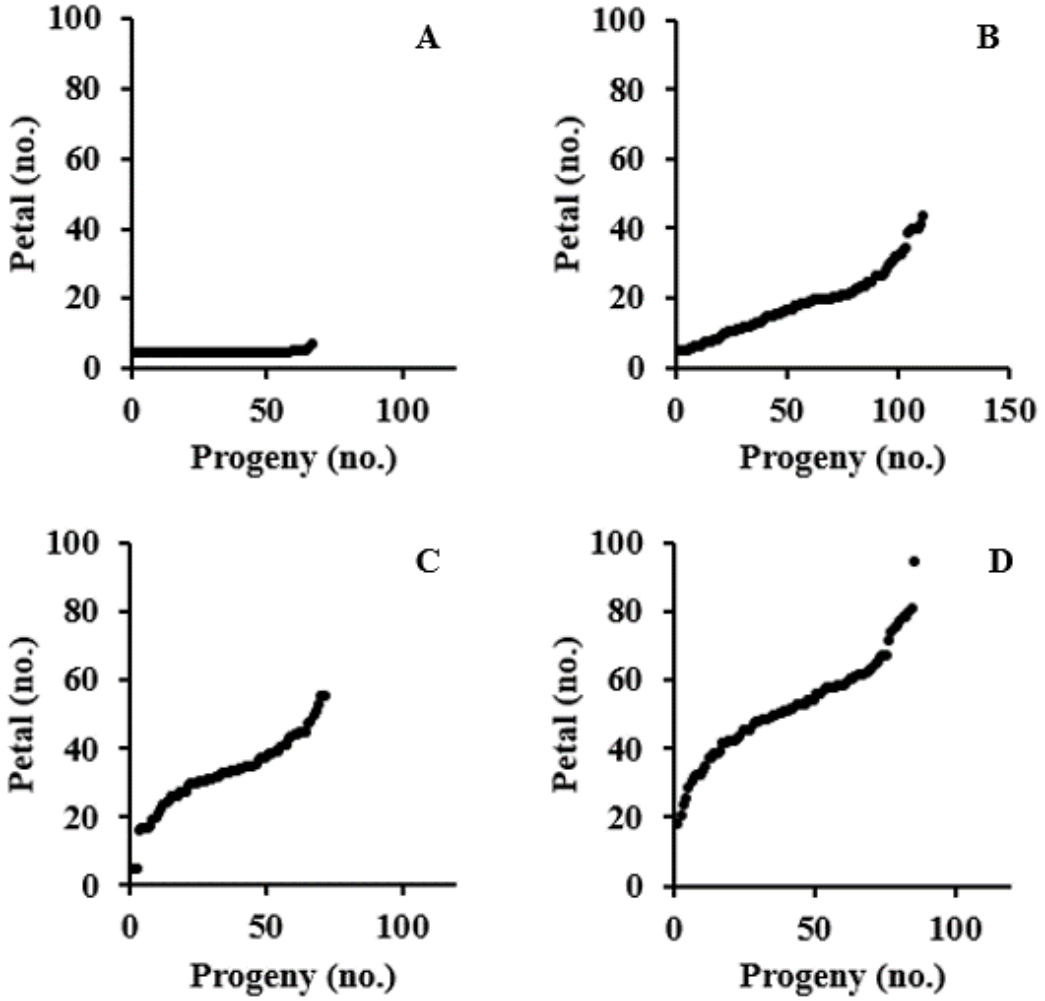

Fig. 8.12. Petal number segregation in progeny from different flower form crosses in *Hibiscus syriacus*. (A) Self-pollination of single-flowered 'Woodbridge' resulting in all single-flowered progeny. (B) Double-flowered Blue Chiffon™ crossed with single-flowered 'Diana' resulting in progeny with a continuous distribution of petal number. (C) Self-pollination of double-flowered White Chiffon® resulting in progeny with a continuous distribution of petal number. (D) Self-pollination of double-flowered Pink Chiffon® resulting in progeny with a continuous distribution of petal number.

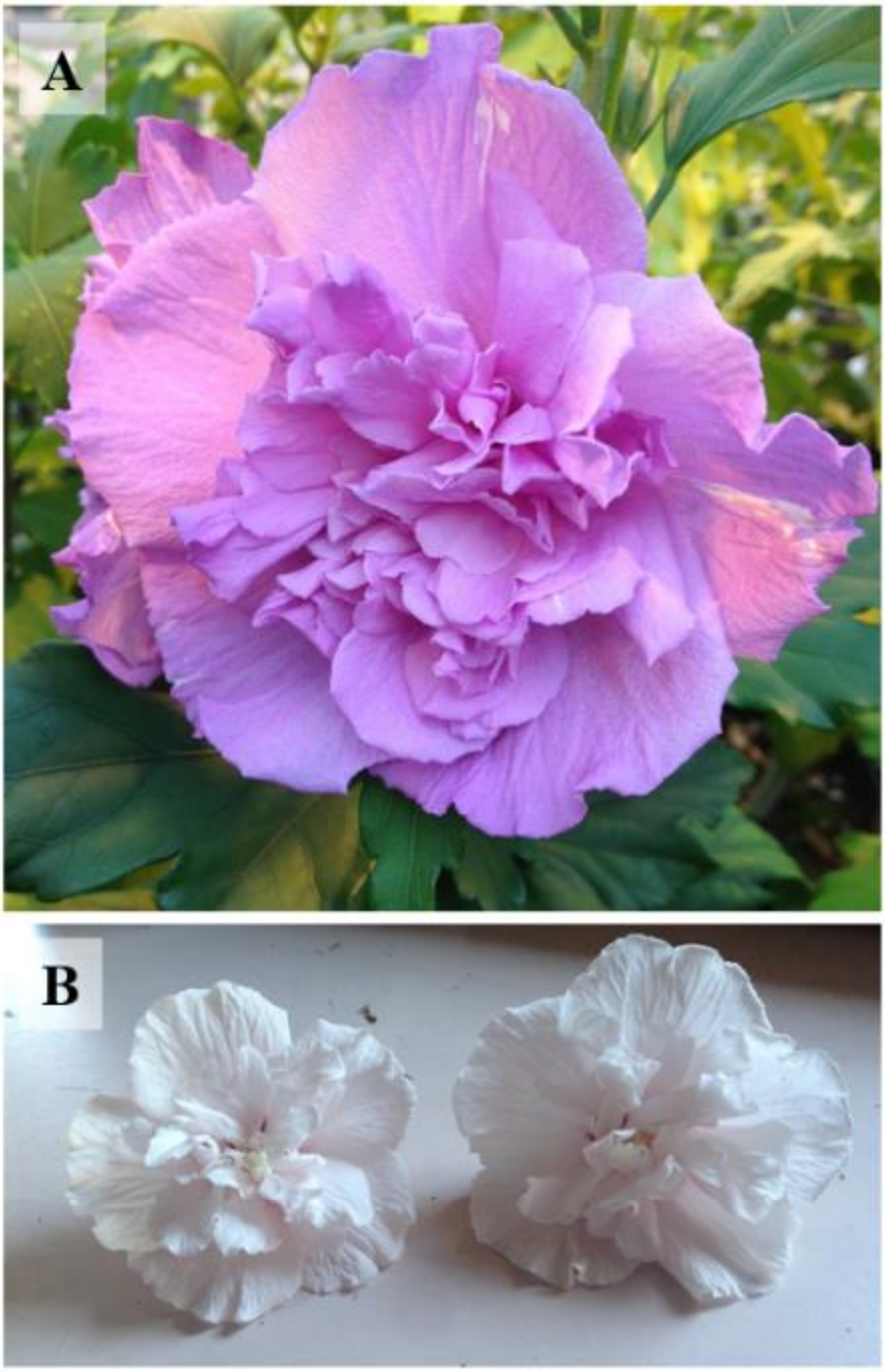

Fig. 8.13. Increased petal number in double-flowered x double-flower crosses in *Hibiscus syriacus*. (A) F_1 hybrid from the cross *H. syriacus* Blue Chiffon™ x *H. syriacus* White Chiffon®. (B) F_1 hybrids from the cross *H. syriacus* White Chiffon® x Strawberry Smoothie™.

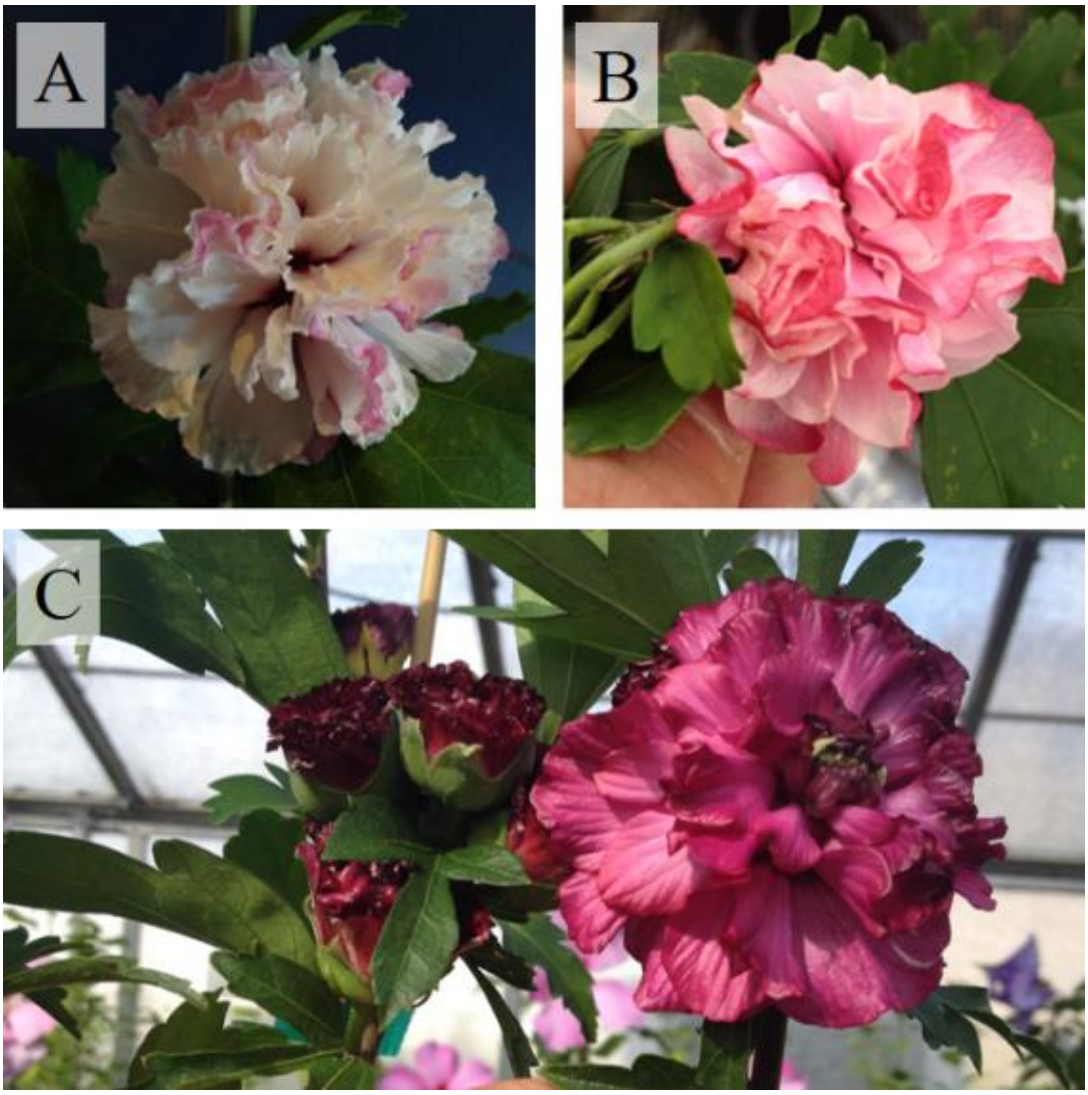

Fig. 8.14. Heritable full-double phenotype observed in F_1 hybrids of *Hibiscus syriacus* 'Blushing Bride'. (A-B) F_1 hybrids from the cross *H. syriacus* 'Blushing Bride' x *H. syriacus* Fiji™ exhibiting a full double flower and bicolor petals. (C) F_1 hybrid from the interploid (4*x* x 6*x*) cross *H. syriacus* 'Blushing Bride' x *H. syriacus* 'Pink Giant' exhibiting dark pink, full double flowers.

Fig. 8.15. Increased flower size in F_1 seedlings from crosses between single-flowered and double-flowered taxa of *Hibiscus syriacus*. (A) Increase petal width in F_1 seedling of Lil' Kim™ crossed with Blue Chiffon™. (B) A comparison of the large flowers of hexaploid *H. syriacus* 'Pink Giant' (above) with an F1 seedling from Lil' Kim™ crossed with Blue Chiffon™.

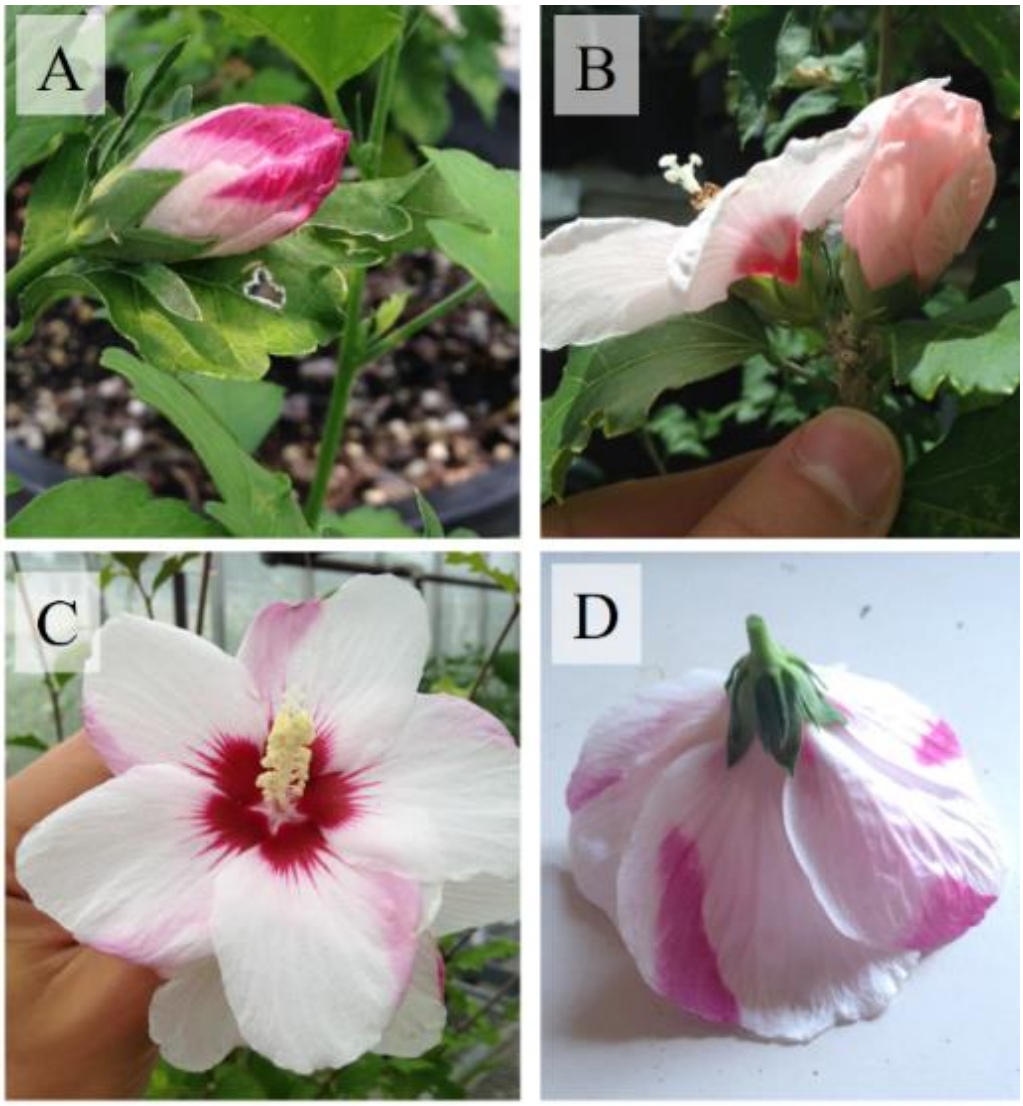

Fig. 8.16. Heritable blush and bicolor phenotype observed in F_1 hybrid seedlings of *Hibiscus syriacus* Fiji™. (A) Bicolor flower bud. (B) Blush flower bud. (C) Adaxial surface of bicolor flower. (D) Adaxial surface of bicolor flower bud.

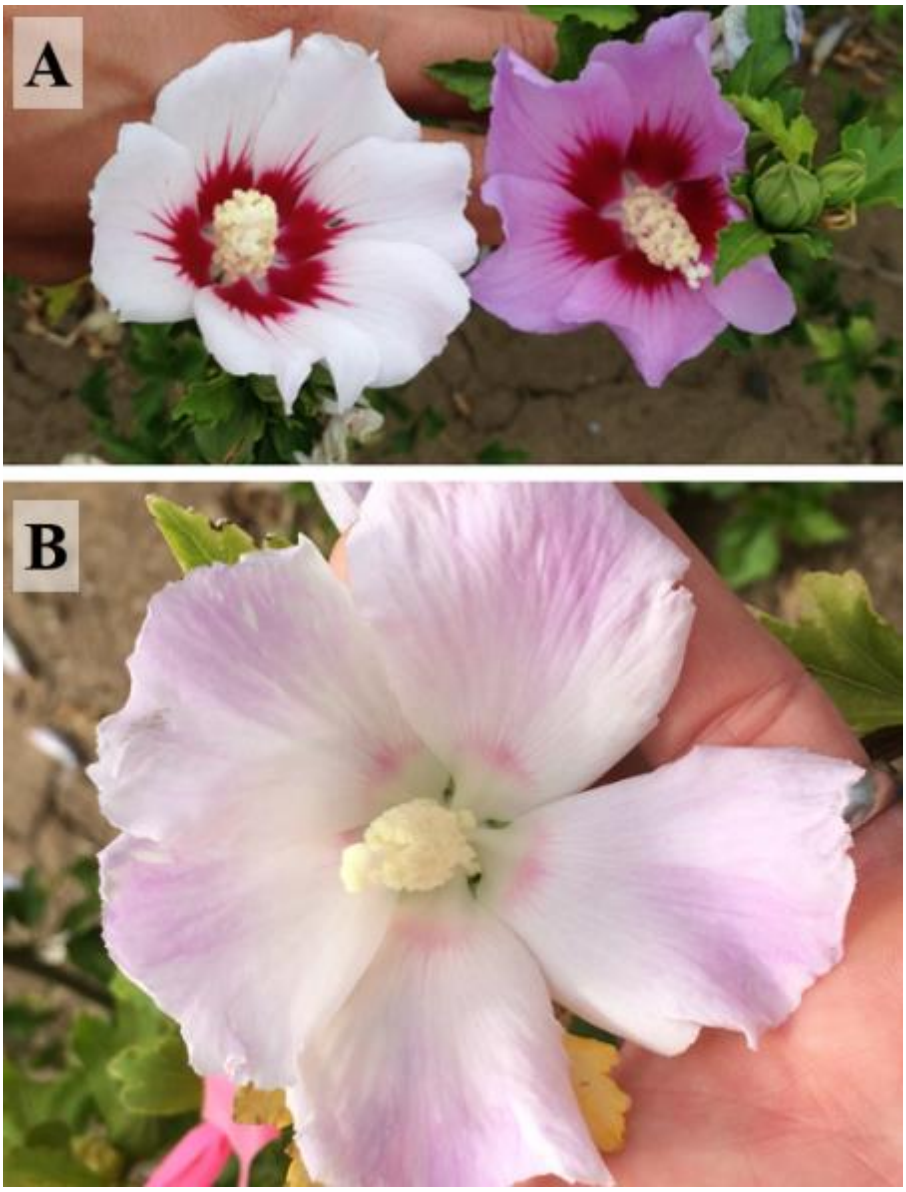

Fig. 8.17. Rare branch sport mutations observed in *Hibiscus syriacus*. (A) *Geisha* branch produced on pink flowered F_1 hybrid. (B) *Geisha* branch produced on pink-flowered F_1 seedling from the cross 'Minerva' x Blue Satin®.

Fig. 8.18. Heritable enlarged stem phenotype observed in F_1 seedlings of *Hibiscus syriacus* 'Buddha Belly'. Left: Enlarged stem in an F_1 hybrid from the reciprocal cross Blue Satin® x 'Buddha Belly'. Right: normal stem in seedling of *H. syriacus*.

CHAPTER 9: GENERAL SUMMARY

Lilacs and hardy hibiscus represent spring and summer ornamental shrubs that beautify the landscape and contribute to the nursery industry. Unlike many agriculture crops, ornamental plant breeders often lack vital information on elite taxa. Our cross-compatibility study represents the largest known crossing study for lilacs. Our research revealed compatible crosses among elite taxa in intraspecific crosses, as well as compatible wide crosses among species. The discovery of interseries crosses that produce large numbers of fruit and seed provide a foundation for attempting green seed germination and embryo rescue in future research, particularly between series Pubescentes and Villosae. Even a handful of true interseries hybrids could provide future lilac breeders with new bridge species for crossing among series.

Cross-compatibility is not only a factor of genetic distance between parents, genome size and ploidy level can also play a role in cross-compatibility. For a genus with many previous reports of polyploid induction, no previous large-scale studies on genome size and ploidy levels existed for lilac prior to our work. Our study represents the most comprehensive genome size and ploidy level survey to date for the genus. The documentation of three fertile triploids (‘Aucubaefolia’, ‘Agincourt Beauty’, and ‘President Grévy’) as well as a diploid taxon that produces unreduced pollen grains (‘Sensation’) will allow breeders to create new populations of interploid hybrids with novel ornamental characteristics. The recovery of a near tetraploid seedling from a cross between diploid ‘Sensation’ and triploid ‘President Grévy’ indicates that increased ploidy may provide a viable mechanism for recovering

seedlings from combinations that are incompatible at the diploid level. Future polyploid induction experiments will be performed to recover polyploid forms of elite taxa for future cross-compatibility studies. Future work on pollen screening will likely reveal more cultivars that produce unreduced pollen for interploid hybridization.

For woody taxa such as lilac, recovering viable seedlings is only half of the battle in new cultivar development. Long generation times limit the amount of progress that can be made over the life of a breeding program. From seed to first flower takes between three to five years in lilac. Our study on green seed germination may provide a production technique that shortens the generation time. Even reducing the generation time by a year could have huge impacts on cultivar development over the lifetime of a breeding program. In addition to shortening the period of juvenility, making selections at the seedling stage for important ornamental traits could reduce the amount of time and field space required to grow and maintain large seedling populations. Developing genetic markers for woody ornamentals, such as lilac, could greatly benefit future breeders. To begin this process, we have developed the first preliminary genetic linkage maps for parents of a biparental mapping population segregating for disease resistance and summer reblooming. Our preliminary maps are still a work in progress, and future efforts at graphical genotyping and deep sequencing will help improve the maps. Future work will also involve construction of a draft genome and identification of novel microsatellite sequences to add to the current maps. Our long-term goal is to produce a high quality consensus map for

dwarf lilacs, phenotype our mapping population for disease resistance and summer reblooming, and to develop genetic markers associated with important traits for lilac.

Like lilacs, little was known about variation in genome size and ploidy levels, as well as cross-compatibility, in Hibiscus syriacus prior to our research. Through our work, we confirmed previously reported hexaploid cultivars available on the market, and are the first to report a fertile, double-flowered hexaploid (Raspberry Smoothie®). This cultivar represents a new possibility for breeding novel pentaploid hybrids with reduced fertility and increased production of petaloid stamen. Fertility tests in H. syriacus confirmed that hexaploid and pentaploid cultivars, as well as production of petaloid stamens, contribute to reducing fertility and weediness in hybrid seedlings. We are also the first to report a cytochimera (Peppermint Smoothie®) among elite cultivars available in the market. Future work will investigate pollen of this cytochimera to determine if the LII histogenic layer (including gametes) are octaploid or tetraploid. Octaploid pollen could lead to novel, fully double, hexaploid cultivars. We also reported ploidy variation among the USNA cultivars, revealing the need for future ploidy testing of nursery materials. In the absence of a flow cytometer, we found that stomata can be a useful tool for separating tetraploids from higher ploidy levels. We are also the first to confirm a wide ploidy series for H. syriacus using a combination of flow cytometry, stomata measurements, and fluorescent in situ hybridization. Having a wide ploidy series in a woody taxon will allow future research to investigate the physiological effects of increased ploidy, particularly on the plant's response to abiotic stress. Previous studies have hypothesized changes in drought response, cold-hardiness,

photosynthetic efficiency, and more in response to ploidy changes in woody taxa. The ploidy series developed in H. syriacus will allow us to test many of these hypotheses in the coming years.

Topping the list of the most important traits for hibiscus breeders are the variable flower phenotypes. However, little information exists on inheritance patterns of traits such as eyespot presence, flower color, and flower form. Our study discovered that eyespot presence is controlled by a single locus called spotless with the homozygous recessive genotype (ss) resulting in a complete knockout of flower color. We also discovered that petal body color is controlled by another locus called geisha (under epistatic control by spotless) with the homozygous recessive genotype (gg) resulting in flowers with eyespots and petal bodies that lack pigment or have a blush pink pigment. Having information about the genetic control of flower colors will allow future breeders to predict the phenotypes of hybrid seedlings and genotype existing elite cultivars. For instance, all blue-flowered cultivars tested are heterozygous for spotless (Ss), carrying at least one recessive allele. Future work will focus on developing populations of true breeding, blue-flowered hybrids. Using recessive testcrosses, seedlings that carry the deleterious spotless allele will be removed and the remaining seedlings intercrosses. Advanced selections could then be crossed to the blue-flowered, hexaploid Azurri Satin® to reduce fertility and deepen the blue pigment in the resulting interploid hybrids.

The results of this dissertation have contributed to the scientific knowledge on two important woody ornamental taxa, and will likely have an impact on future breeders of lilac and hardy hibiscus. Many of these projects have resulted in novel

seedlings that are currently being trialed, clonally propagated, and considered for future release. In addition, many seedlings will be incorporated into future crosses and future research projects. Therefore, the work began in this dissertation will hopefully continue to make contributions to science and to the nursery industry for many years to come.

APPENDIX: IMPROVED METHOD OF ENZYME DIGESTION FOR ROOT TIP CYTOLOGY

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APPENDIX: IMPROVED METHOD OF ENZYME DIGESTION FOR ROOT TIP CYTOLOGY

Additional index words: chromosome counts, root squash, ploidy, ultraviolet resin, carbol fuchsin stain

Abstract: Chromosome numbers are an important character for multiple fields of plant sciences, from plant breeding and genetics to systematics and taxonomy. Accurate chromosome counts in root tips of woody plants are often limited by their small, friable roots with numerous, small chromosomes. Current hydrolysis and enzyme digestion techniques require handling of roots prior to the root squash. However, optimum chromosome spread occurs when the cell walls have degraded past the point of easy handling. Here, we present a new enzyme digestion protocol that is fast, efficient, and flexible. This protocol reduces handling of the roots allowing for long duration enzyme digestion. Digestions are performed on a microscope slide, eliminating the need for handling digested cells with forceps or pipettes. To illustrate the flexibility of this method across woody plant taxa, we performed chromosome counts on five angiosperms and one gymnosperm. Ploidy levels included diploids, triploids, and tetraploids with chromosome numbers ranging from $2n = 16$ to $2n = 80$. The range of holoploid 2C genome sizes spanned 1.54 pg to 24.71 pg. This protocol will provide a useful technique for plant cytologists working with taxa that exhibit a wide range of genome size and ploidy levels.

Introduction

Genome size, chromosome number, and ploidy level are important biological parameters for plant breeding, systematics, and evolution. Since the first published chromosome counts of plants in 1882 (Garbari et al., 2012), approximately 25% of angiosperms have been measured for chromosome number (Castiglione and Cremonini, 2012) and 2.1% have measurements of genome size (Garcia et al., 2014). Holoploid 2C genome sizes of plants span approximately a 2400-fold range, from 0.13 pg (*Genlisea margaretae* Hutch.) to 304.46 pg (*Paris japonica* Franch.) (Bennett and Leitch, 2011; Chen et al., 2014; Fleischmann et al., 2014; Pellicer et al. 2010).

Traditional cytology has many uses in modern studies of woody plants. Combined with flow cytometry, cytology has been used to calibrate genome size with ploidy level, and to confirm chromosome and ploidy variation among related taxa and hybrids in both temperate woody species (Contreras et al., 2007, 2009, 2010; Gillooly and Ranney, 2015; Jones et al., 2007; Lattier et al., 2013; Oates et al., 2014; Parris et al., 2010; Ranney et al., 2007; Rothleutner et al., 2016; Rounsaville and Ranney, 2010; Shearer and Ranney, 2013) and tropical woody species (Bationo-Kando et al., 2016; Cai et al., 2013; Dahmer et al., 2009; Schneider et al., 2015). Traditional cytology can be used for identifying aneuploids (additional or missing chromosomes) (Hu et al., 2015) or dysploids (alterations from chromosome fusion or fission) that are difficult to detect using flow cytometry (Rockinger et al., 2016). To meet the demand for accurate chromosome counts, traditional cytology has proven useful in the development of databases across taxonomic groups and formation of data sets for

meta-analysis of chromosome number across plants and animals (Peruzzi et al., 2014).

Although traditional cytology has been an invaluable tool for studies in plant genetics, cytology on woody plants can be challenging compared to their herbaceous counterparts. In general, many woody plants possess small, friable roots with numerous small chromosomes making cytology particularly difficult (Lattier et al., 2013). Chromosome counting techniques can be tedious and require experienced histologists (Ochatt, 2008). In the modern era, cytogenetic studies in hardwood trees have not kept pace with current genomic studies due to small genomes and relatively small chromosomes (Ribeiro et al., 2008). Traditional cytology is also necessary for chromosomal fluorescent labeling techniques, such as FISH (fluorescent in situ hybridization) and GISH (genomic in situ hybridization), and has proven valuable for characterizing hybrids in woody plants with small chromosomes (Van Laere et al., 2010).

Current root tip cytology consists of three broad steps (pre-fixative, fixative, and root squash) with slight variations for each step. For the pre-fixative step, roots are treated with one or more spindle fiber inhibitors to allow the cell cycle to continue to metaphase while arresting cytokinesis. In addition, root tip cold treatments have been used to help arrest cells at metaphase and condense chromosomes (Jauhar, 2003). Next, root tips are fixed in a solution that arrests the cell cycle and then they are stored in an aqueous ethanol solution until observation. For the root squash step, roots are hydrolyzed in hydrochloric acid or a combination of hydrochloric acid and ethanol. Alternatively, cell walls may be broken down by enzyme digestion using

combinations of cellulase, cytohelicase, and pectolyase. Chromosome stains include modified carbol fuchsin, Feulgen, Giemsa, and acetocarmine, as well as fluorochrome stains (Bationo-Kando et al., 2016; Contreras et al., 2010; Jones et al., 2007; Rothleutner et al., 2016; Schneider et al., 2015).

In our observations, to obtain the highest quality chromosome spread, cell wall degradation often must proceed beyond the point which the root tip can be easily handled and still remain intact. However, most protocols using enzyme digestion require handling after hydrolysis or digestion. This creates a problem that can be solved by digesting the excised root tip on the same surface used to perform the root squash. Once the cell walls have been fully digested, the weight of the cover slip on the root tip should be sufficient to initially spread the cells. In the current study, we report a novel root squash protocol from the Ornamental Plant Breeding Laboratory at Oregon State University that has proven to be a fast, effective, and adjustable root tip cytology method applicable across a wide range of woody plant taxa.

Methods and Materials

Plant Material. Six taxa were investigated to represent a wide range of genome size and chromosome number, including five angiosperms and one gymnosperm (Table A.1). Plants were grown in a temperature-controlled glasshouse at Oregon State University. Although variable growth conditions would likely affect the quality of roots for cytology, all plants were grown under the same standard glasshouse conditions. Plants were container-grown in a 2:1 mixture of Metro-Mix Professional Growing Mix (Sun Gro Horticulture, Agawam, MA) and Perlite

(Supreme Perlite Company, Portland, OR). Plants were initially hand-watered using municipal water on an as-needed basis and substrate solution pH and electrical conductivity (EC) were routinely monitored using the pour-through nutrient extraction procedure (Wright, 1986). Once the substrate solution EC was less than EC = 1.0 uS/cm, plants were fertigated at each irrigation with Peters Professional 20N–4.4P–16.6K plus micronutrients (Everris NA Inc., Dublin, OH), calibrated such that irrigation water EC = 1.0 uS/cm. Plants were grown in a glasshouse with set temperatures of 24 °C day/ 17 °C night and a 14-h photoperiod.

Reported chromosome numbers of the taxa investigated span a range of $2n$ = 16 to $2n$ = 80 and holoploid genome sizes of 1.54 pg to 24.71 pg. *Ribes sanguineum* Pursh is reported to be a diploid ($2n = 2x = 16$) (Darlington and Wylie, 1956) with a holoploid genome size of 1.94 pg (OPBL, unpublished data). Roots were collected from an open-pollinated seedling of *R. sanguineum* 'Pokey's Pink'. *Quercus robur* L. is reported to be a diploid ($2n = 2x = 24$) with a holoploid genome size of 1.85 pg (Favre and Brown, 1996). Roots were collected from an open-pollinated seedling of the columnar *Q. robur* 'Fastigiata'. *Thuja occidentalis* L. is reported to be a diploid ($2n = 2x = 22$) with a holoploid genome size of 24.71 pg (Hizume et al., 2001). Roots were sampled from an open-pollinated seedling collected from a plant growing at the Lewis Brown Horticulture Research Farm in Corvallis, OR. *Cercidiphyllum japonicum* Siebold & Zucc. is reported to be a diploid ($2n = 2x = 38$) with a holoploid genome size of 1.53 pg (Garcia et al., 2010). Roots were collected from an open-pollinated seedling of *C. japonicum* 'Rotfuchs' (Red Fox) at the U.S. National Arboretum (Beltsville, MD). *Acer tataricum* subsp. *ginnala* (Maxim.) Wesm. is

reported to be both a diploid ($2n = 2x = 26$) (Darlington and Wylie, 1956) with a holoploid genome size of 1.65 pg (Lattier, 2016) and an induced autotetraploid ($2n = 4x = 52$) with a holoploid genome size of 3.10 pg (Lattier, 2016). Roots were collected from an open-pollinated seedling (OP2016-04-014) of a tetraploid cytotype (12-0011-010) in an isolation block (field location 75.18) comprised of a mixture of tetraploid and diploid cytotypes. *Hibiscus syriacus* L. is reported to be a tetraploid ($2n = 4x = 80$) (Darlington and Wylie, 1956) with a holoploid genome size of 4.70 pg (Contreras et al., 2013). Roots were collected from a self-pollinated seedling of *H. syriacus* 'Notwoodtwo' White Chiffon™.

Pre-fixative. Root tips were collected before 1000 HR following two sunny days and suspended in 1.5 mL microcentrifuge tubes containing a solution of 2mM 8-hydroxyquinoline + 0.24 mM cycloheximide. Root tips were treated in the dark at room temperature for 2.5 h before a cold period in a refrigerator at 4 °C for 2.5 h.

Fixative. After five hours in the pre-fixative solution, root tips were transferred to a filter paper lined glass funnel atop a Büchner (vacuum) flask. Root tips were thoroughly rinsed with filter sterilized water, and fixed overnight at room temperature in Carnoy's solution (6 parts 95% ethanol: 3 parts chloroform: 1 part glacial acetic acid; by volume). After fixing the cells, roots were transferred to a storage solution of 70% ethanol and stored in a refrigerator at 4 °C.

Root Squash. Roots were transferred from storage solution to a small beaker of filter sterilized water for five minutes, swirling occasionally. This step was repeated twice for a triple rinse of each root over a total of 15 min. Each root was placed on a clean slide under a dissecting microscope (SMZ1500; Nikon, Tokyo,

Japan) and the root tip was excised (Fig. A.1A). The remaining root was discarded and excess water was removed with a single-ply, low lint tissue (VWR International; Radnor, PA) leaving only a small droplet encompassing the root tip to maintain hydration. To localize enzyme digestion to the microscope slide, a circular well was created using an ultraviolet resin pen (Bondic®; Niagara Falls, NY). A circle of ultraviolet resin was drawn around the root tip and water droplet (Fig. A.1B). To facilitate removal of the circular well, a "pull tab" of ultraviolet resin was placed over a small piece of wax paper (Fig. A.1B). The slide was then placed in an ultraviolet crosslinker (CL 1000 UV Crosslinker; UVP, LLC, Upland, CA) for 30 s to set the ultraviolet resin. Although a crosslinker was used, UV lamps and flashlights are ubiquitous and can be acquired for less than twenty dollars, making the price of this protocol comparable to previous methods. Using a low lint tissue, the droplet of water containing the root tip was wicked away and a droplet of enzyme solution was pipetted on the root tip (Fig. A.1B). The enzyme solution was composed of 0.5% cellulase (from *Trichoderma reesei*; Sigma, St. Louis, MO), 0.5% cytohelicase (from *Helix pomatia*; Sigma) and 0.5% pectolyase (from *Aspergillus japonicus*; Sigma) in a sodium citrate buffer at pH = 4.5.

Next, the slide was placed in a humid environment to maintain the droplet throughout the digestion period. Humidity was maintained in a glass Petri dish with dampened filter paper; the slide was kept dry by resting it on a small weighing dish (Fig 1C). The glass Petri dish containing the slide was incubated at 37 °C in an oven (Isotemp® Oven 655F; Thermo Fisher Scientific, Waltham, MA). Multiple root tips per taxa were digested at varying durations until optimal digestion was achieved.

Optimal digestion was achieved when the weight of the cover slip provided enough force to break apart the root tip. Less than ten slide preparations per taxa were required to find an optimal digestion time for each taxon.

Once digestion was complete, the glass Petri dish and slide were removed from the oven. To prepare the root squash, the paper "pull tab" was used to remove the ring of UV resin (Fig. A.1D). Excess enzyme solution was wicked away before adding a drop or two of filter sterilized water to the root tip to rinse. The area surrounding the root tip was wiped clean with a low lint tissue before wicking away excess water on the surface of the root tip. A single drop of modified carbol fuchsin stain (Kao, 1975) was pipetted on the root tip (Fig. A.1E). Using half of a double-sided razor blade, a bridge was made to position a 22 × 22 mm cover slip at an angle over the root tip and stain droplet (Fig. A.1E). The cover slip was quickly lowered on to the slide to prevent bubbles and covered with a sheet of bibulous paper (Fig. A.1F). Pressure was applied to the cover slip using a pencil eraser while the bibulous paper wicked away excess stain (Fig. A.1F). Slides mounted with root squashes were placed back into petri dishes and stored at room temperature. This allowed for multiple slide preparations without sealing since the humidity chambers prevented the slides from drying (Fig. A.1G).

Chromosome Counts. All slides were screened for condensed chromosomes at a magnification of ×200 (×10 adapter and ×20 objective) on a light microscope (Axio imager.A1: Zeiss, Thornnwood, NY; AxioCam MRm, Zeiss). Condensed and spread chromosomes were photographed under oil immersion at ×1000 (×10 adapter and ×100 objective). To maximize resolution of each chromosome and extend depth

of field, multiple photos were taken per cell at different focal distances. Bulk focus stacking was performed using the Auto Blend feature in Photoshop CC 2015.5.1 (Adobe Systems; San Jose, CA). Bulk focus stacking of small chromosomes can still leave individual chromosomes out of focus. Therefore, fine editing (selecting sharpest focus for individual chromosomes) was performed when necessary using GIMP 2.8.18 (GNU Image Manipulation Program, https://www.gimp.org/). A minimum of 15 highly resolved cells were observed per taxa.

Results and Discussion

Root squashes were successful across all six taxa. All roots were treated the same in the pre-fixative and fixative steps. However, we recommend adjusting duration of pre-fixative treatment to each specific taxon, as recommended in other woody plant cytology protocols (Gamage and Schmidt, 2009). Since the duration of the mitotic cycle is positively correlated with genome size, a longer pre-fixative process should be used as genome sizes increase (Bennett, 1998; Schneider et al. 2015). Duration of digestion was adjusted for each taxon (Table A.1). We observed that digestion time was influenced by root tip size and genome size of each taxa. Optimal digestion times were taxa-specific and varied from 30 min for *Ribes sanguineum* to 3-h for *Thuja occidentalis* (Table A.1). When calibrating the digestion step, under-digested root tips failed to break apart under the weight of the cover slip while over-digested root tips yielded cells with missing and far-spread chromosomes. Modified carbol fuchsin proved a fast and effective stain requiring no extra steps,

such as heating the slide (required for acetocarmine), extended incubation (required for Fuelgen), or special light sources (required for fluorochrome stains).

Chromosome counts of metaphase cells revealed diploid, triploid, and tetraploid cytotypes (Fig. A.2). *Ribes sanguineum* was confirmed as a diploid ($2n = 2x = 16$) (Fig. A.2A). *Quercus robur* was confirmed as a diploid ($2n = 2x = 24$) (Fig. A.2B). *Thuja occidentalis* was confirmed as a diploid ($2n = 2x = 22$) (Fig. A.2C). *Cercidiphyllum japonicum* was confirmed as a diploid ($2n = 2x = 38$) (Fig. A.2D). *Acer tataricum* subsp. *ginnala* was confirmed as a triploid ($2n = 3x = 39$) resulting from an interploid cross in our isolation block (Fig. A.2E). *Hibiscus syriacus* was confirmed as a tetraploid ($2n = 4x = 80$) (Fig. A.2F).

With chromosome estimates published for only 25% of angiosperms (Castiglione and Cremonini, 2012), there is a clear need for additional cytological studies. Chromosome counts are an important biological character necessary to multiple fields of plant sciences, from plant breeding and genetics to systematics and taxonomy. Our protocol provides an improved method for root tip cytology that may contribute to the growing number of chromosome surveys. The new enzyme digestion protocol proved an effective tool for producing high resolution metaphase chromosomes across multiple woody plant taxa. Accurate chromosome counts are critical when assessing wide hybrids or interploid crosses. Confirmation of a triploid cytotype in *Acer tataricum* subsp. *ginnala* illustrates the utility of the protocol in confirming hybrids from interploid crosses.

Minimal handling of root tips combined with long duration digestion makes this protocol a practical method for root tip cytology in woody plants. Its simplicity

makes it a fast method for producing quality root squashes. Other hydrophobic barrier pens (PAP pens) have been used for cytology. However, UV resin pens are less expensive and leave little to no residue, unlike PAP pens which require xylene for residue removal. Its flexibility can be combined with other methods with more complicated procedures, such as DAPI-staining, to increase resolution for plants with high ploidy and numerous chromosomes.

Literature Cited

Bationo-Kando, P., J.-D. Zongo and S. Siljak-Yakovlev. 2016. First genome size assessment, heterochromatin and rDNA chromosome mapping in the genus *Sclerocarya* (Anacardiaceae): insight into the new basic chromosome number. Bot. Lett. 163:11-17.

Bennett, M.D. 1998. Plant genome values: how much do we know? Proc. Natl. Acad. Sci. USA 95:2011-2016.

Bennett, M.D. and I.J. Leitch. 2011. Nuclear DNA amounts in angiosperms: targets, trends and tomorrow. Ann. Bot. 107:467-590.

Cai, Z.Q., T. Zhang and H.Y. Jian. 2013. Chromosome number variation in a promising oilseed woody crop, *Plukenetia volubilis* L. (Euphorbiaceae). Caryologia 66:54-58.

Castiglione, M.R. and R. Cremonini. 2012. A fascinating island: 2*n* = 4. Plant Biosyst. 146:711-726.

Contreras, R.N., T.G. Ranney and S.P. Tallury. 2007. Reproductive behavior of diploid and allotetraploid *Rhododendron* L. 'Fragrant Affinity'. HortScience 42:31-34.

Contreras, R.N., J.M. Ruter and W.W. Hanna. 2009. An Oryzalin-induced autoallooctaploid of *Hibiscus acetosella* 'Panama Red'. J. Am. Soc. Hort. Sci. 134:553-559.

Contreras, R.N., J.M. Ruter and B.M. Schwartz. 2010. Oryzalin-induced tetraploidy in *Cryptomeria japonica* (Cupressaceae). HortScience 45:316-319.

Contreras, R.N., M. Friddle and J.D. Lattier. 2013. Relative fertility and ploidy levels of selected rose-of-sharon cultivars. Proc. 58th Ann. SNA Res. Conf. 58:232-236.

Chen, S.-C., C.H. Cannon, C.-S. Kua, J.-J. Liu and D.W. Galbraith. 2014. Genome size variation in the Fagaceae and its implications for trees. Tree Genet. & Genomes 10:977-988.

Darlington, C.D. and A.P. Wylie. 1956. Chromosome atlas of flowering plants. The Macmillan Company, New York.

Dahmer, N., M.T.S. Wittmann and P.E. Kaminski. 2009. Chromosome number and karyotype of the endangered Amazonian woody *Centrolobium paraense* Tul. species. Crop Breed. Appl. Biotechnol. 9:382-385.

Favre J.M. and S. Brown. 1996. A flow cytometric evaluation of the nuclear DNA content and GC percent in genomes of European oak species. Ann. Sci. For. 53:915-917.

Fleischmann A., T.P. Michael, F. Rivadavia, A. Sousa, W. Wang, E.M. Temsch, J. Greilhuber, K.F. Müller and G. Heubl. 2014. Evolution of genome size and chromosome number in the carnivorous plant genus *Genlisea* (Lentibulariaceae), with a new estimate of the minimum genome size in angiosperms. Ann. Bot. 114:1651–1663.

Gamage, H.K. and S. Schmidt. 2009. Short Communication. A robust method for chromosome quantification and ploidy determination in woody species. Aust. J. Bot. 57:87-93.

Garbari, F., G. Bedini and L. Peruzzi. 2012. Chromosome numbers of the Italian flora. From the Caryologia foundation to present. Caryologia 65:62-71.

Garcia, S., T. Garnatje, O. Hildalgo, G. Mas de Xaxars. J. Pellicer, I. Sánchez-Jiménez, D. Vitales and J. Vallès. 2010. First genome size estimations for some eudicot families and genera. Collect. Bot. 29:7-16.

Garcia, S., I.J. Leitch, A. Anadon-Rosell, M.Á. Canela, F. Gálvez, T. Garnatje, A. Gras, O. Hidalgo, E. Johnston, G.M. de Xaxars, J. Pellicer, S. Siljak-Yakovlev, J. Vallès D. Vitales and M.D. Bennett. 2014. Recent updates and developments to plant genome size databases. Nucleic Acids Res. 42 (D1):D1159–D1166.

Gillooly, D.A. and T.G. Ranney. 2015. Genome size and ploidy Levels in the genus *Kalmia*. HortScience 50:1426-1428.

Hizume M, T. Kondo, F. Shibata and R. Ishizuka. 2001. Flow cytometric determination of genome size in the Taxodiaceae, Cupressaceae *sensu stricto* and Sciadopityaceae. Cytologia 66:307-311.

Hu, F.R., H.H. Liu, F. Wang, R.L. Bao and G.X. Liu. 2015. Root tip chromosome karyotype analysis of hyacinth cultivars. Genet. Mol. Res. 14:10863-10876.

Jauhar, P.P. 2003. Haploid and doubled haploid production in durum wheat by wide hybridization, p. 161-166. In: M. Maluszynski, K.J. Kasha, B.P. Forester and I. Szarejko (eds.). Doubled haploid production in crop plants - a manual. Kluwer Academic Publishers, Boston.

Jones, J.R., T.G. Ranney and N.P. Lynch. 2007. Ploidy levels and genome sizes of diverse species, hybrids, and cultivars of rhododendron. J. Am. Rhod. Soc. 61:220-227.

Kao, K.N. 1975. A nuclear staining method for protoplasts, p. 60-64. In: O.L. Gamborg and L.Z. Wetter (eds.) Plant tissue culture methods. L.R. National Research Council of Canada. Prairie Regional Laboratory. Saskatoon, Saskatchewan, Canada.

Lattier, J.D., T.G. Ranney and N.P. Lynch. 2013. History and cytological reassessment of *Rhododendron canadense*. J. Am. Rhod. Soc. 67:92-98.

Lattier, K.S. 2016. Inducing and evaluating phenotypic and cytometric variation in landscape plants: Observations from *Acer*, *Ornithogalum*, and *Penstemon*. Oregon State University, Corvallis, M.S. Thesis.

Oates, K.M., T.G. Ranney and D.H. Touchell. 2014. *Campsis* ×*tagliabuana* 'Chastity': A highly infertile triploid trumpet vine. HortScience 49:343-345.

Ochatt, S.J. 2008. Flow cytometry in plant breeding. Cytometry 73:581-598.

Parris, J.K., T.G. Ranney and W.V. Baird. 2010. Ploidy levels, relative genome sizes, and base pair composition in magnolia. J. Am. Soc. Hortic. Sci. 135:533-547.

Peruzzi, L., K.F. Caparelli and G. Bedini. 2014. A new index for the quantification of chromosome number variation: An application to selected animal and plant groups. J. Theor. Biol. 353:55-60.

Pellicer J., M.F. Fay and I.J. Leitch. 2010. The largest eukaryotic genome of them all? Bot. J. Linn. Soc. 164:10–15.

Ranney, T.G., N.P. Lynch, P.R. Fantz and P. Capiello. 2007. Clarifying taxonomy and nomenclature of *Fothergilla* (Hamamelidaceae) cultivars and hybrids. HortScience 42:470-473.

Ribeiro, T., A Barão, W. Viegas and L. Morais-Cecílio. 2008. Molecular cytogenetics of forest trees. Cytogenet. Genome Res. 120:220-227.

Rockinger, A., A. Sousa, F.A. Carvalho and S.S. Renner. Chromosome number reduction in the sister clad of *Carica papaya* with concomitant genome size doubling. Am. J. Bot. 103:1082-1088.

Rothleutner, J.J., M.W. Friddle and R.N. Contreras. 2016. Ploidy Levels, relative genome sizes, and base pair composition in *Cotoneaster*. J. Am. Soc. Hortic. Sci. 141:457-466.

Rounsaville, T.J. and T.G. Ranney. 2010. Ploidy levels and genome sizes of *Berberis* L. and *Mahonia* Nutt. species, hybrids, and cultivars. HortScience 45:1029-1033.

Schneider, J.V., J. Paule, J. Gitaí, S. Dressler, C.L.S. Gusmão and A.M. Benko-Iseppon. 2015. Divergent genome sizes reflect infrafamilial subdivision of the Neotropical woody Marcgraviaceae. Bot. J. Linn. Soc. 177:1-14.

Shearer, K. and T.G. Ranney. 2013. Ploidy levels and relative genome sizes of species, hybrids, and cultivars of dogwood (*Cornus* spp.). HortScience 48:825-830.

Van Laere, K., L. Khrustaleva, J.V. Huylenbroeck and E. Van Bockstaele. 2010. Application of GISH to characterize woody ornamental hybrids with small genomes and chromosomes. Plant Breeding 129:442-447.

Wright, R.D. 1986. The pour-through nutrient extraction procedure. HortScience 21:227-229.

Tables

Table A.1. Digestion times for taxa investigated using an improved enzyme digestion protocol for root tip cytology.

Taxa	Digestion time (h)[z]	2C formula[y]	2C genome size (pg)[x]
Cercidphyllum japonicum 'Rotfuchs' Red Fox	0.5	2*n* = 2*x* = 38	1.53
Ribes sanguineum 'Pokey's Pink'	0.5	2*n* = 2*x* = 16	1.94
Acer tataricum subsp. *ginnala*	1	2*n* = 3*x* = 39	2.34*
Quercus robur	2	2*n* = 2*x* = 24	1.85
Hibiscus syriacus 'Notwoodtwo' White Chiffon™	2	2*n* = 4*x* = 80	4.70
Thuja occidentalis	3	2*n* = 2*x* = 22	24.71

[z]Optimal digestion times for root tip cytology.
[y]Ploidy and chromosome number of somatic root tip cells.
[x]Reported holoploid 2C genome size; * = estimated triploid 2C genome size based on published diploid and tetraploid genome sizes.

Figures

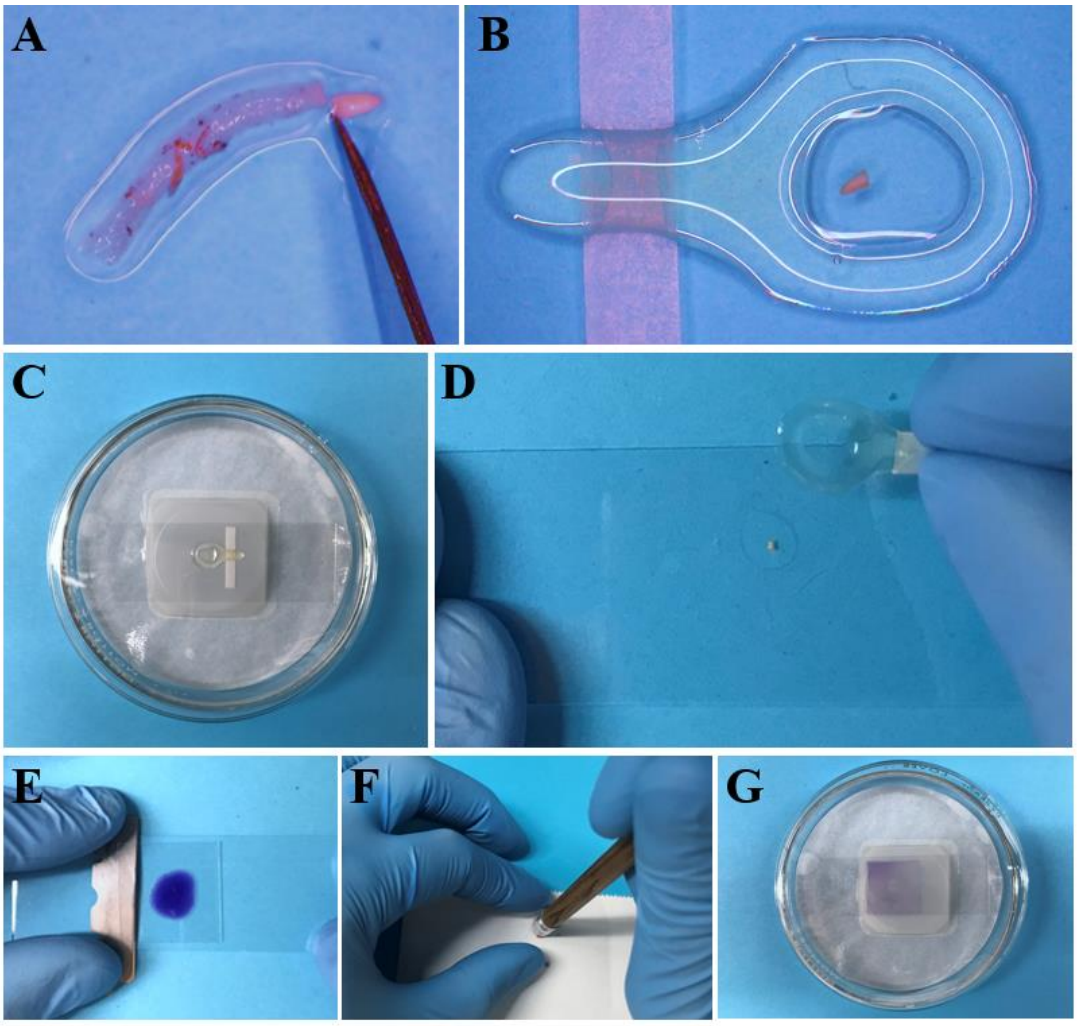

Fig. A.1. Steps in an improved enzyme digestion for root tip cytology. (A) Rinsed roots are placed on a clean slide under a dissecting microscope and root tip is removed. (B) A ring of ultraviolet resin is placed around the root tip and overlapping a waxed paper pull tab. The resin is set in an ultraviolet crosslinker and enzyme digestion solution is added to the well to encompass the root tip. (C) Enzyme digestion takes place in a 37 °C oven with the slide in a petri dish atop a small weighing dish and moist filter paper. (D) After digestion, the pull tab is used to remove the enzyme digestion well and the root tip is rinsed with a droplet of water before being wicked dry. (E) A drop of modified carbol fuchsin stain is added and a glass cover slip is lowered on to the root tip with half of a double-sided razor blade. (F) The slide is covered with bibulous paper and the root is squashed using gentle pressure from a pencil eraser. (G) The prepared slide is stored at room temperature in the petri dish until viewed with a light microscope.

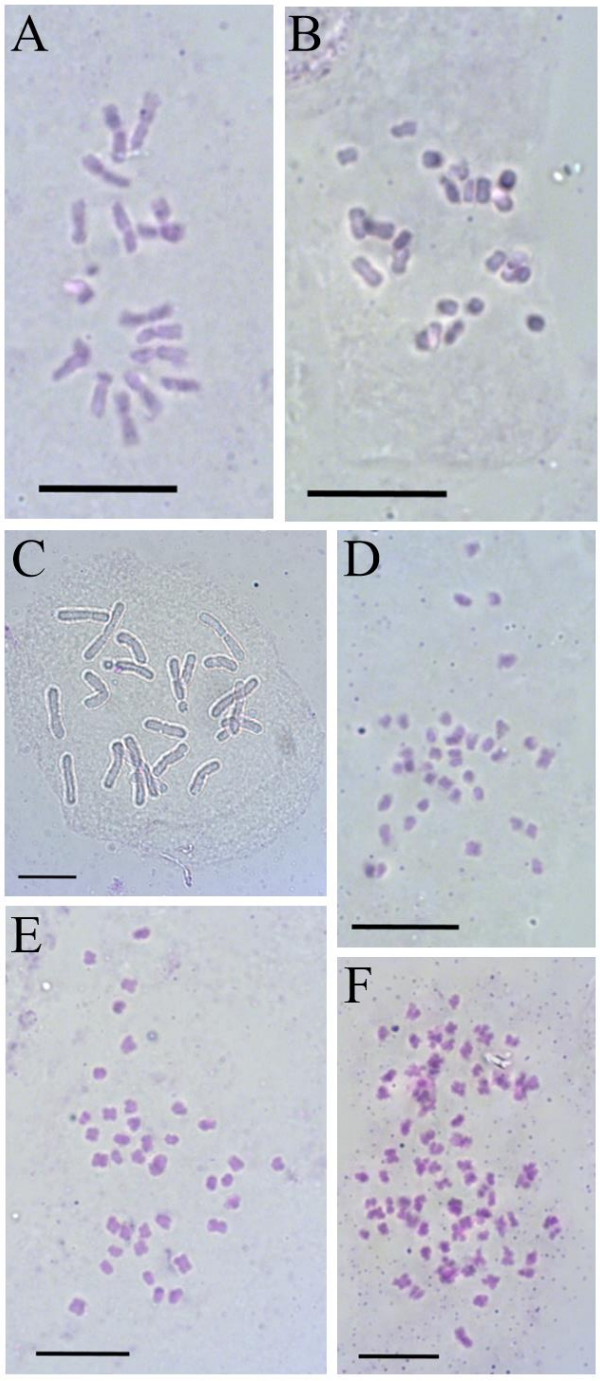

Fig. A.2. Photomicrographs from an improved enzyme digestion protocol for root tip cytology. Metaphase chromosomes observed at magnification ×1000 from root apical meristems. Scalebar = 10 μm. (A) *Ribes sanguineum*, $2n = 2x = 16$; (B) *Quercus robur*, $2n = 2x = 24$; (C) *Thuja occidentalis*, $2n = 2x = 22$; (D) *Cercidiphyllum japonicum*, $2n = 2x = 38$ (E) *Acer tataricum* subsp. *ginnala*, $2n = 3x = 39$; (F) *Hibiscus syriacus*, $2n = 4x = 80$.